HUMAN MESENCHYMAL STEM CELLS FOR TISSUE ENGINEERING BONE

By

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Abstract

My thesis hypothesises that cells isolated from human bone marrow could be stimulated to differentiate into osteoblasts and that, these cells when cultured on a scaffold could be used in the tissue engineering of bone, as the constructs could potentially be implanted into patients' bone defects resulting in increased healing.

The multipotency of the bone marrow-isolated cells was assessed by the use of histological stains to show that they could be stimulated to differentiate into osteoblasts, chondrocytes and adipocytes. The cells were further characterised by Stro-1 and, as a result, were defined as mesenchymal stem cells (MSCs). More specifically, it was shown that when the cells were cultured with osteogenic stimulants, the production of osteoblastic proteins, such as alkaline phosphatase, osteopontin and osteocalcin increased ($P<0.05$), indicating that they were differentiating into osteoblasts. The cells were shown to retain their multipotent potential, and could be manipulated by surfaces and culture supplements *in vitro*.

The ability of MSCs to differentiate in this way is fundamental for their use in tissue engineering of bone. This concept was investigated by growing marrow-isolated human MSCs on 3-dimensional porous hydroxyapatite (HA) scaffolds. In this environment, MSCs were shown to differentiate into osteoblasts, producing extracellular bone matrix proteins, even without the use of osteogenic stimulants.

In order to simulate living bone conditions, where osteoblasts are perfused with tissue fluid, a novel bioreactor was developed for the culture of the MSC-HA constructs. Use of the bioreactor, to perfuse MSCs with oxygenated medium, promoted 3-dimensional growth of cells and stimulated differentiation into active osteoblasts resulting in increased production of extracellular matrix. Furthermore, the flow of medium through the scaffold encouraged the movement of cells and increased penetration into the HA ($P<0.05$).

Cryopreservation was shown to be an effective method of storage, as it did not alter the cell function, measured by proliferation and the ability to differentiate into osteoblasts. Thus, it can be used as a practical method for storage of MSCs between harvest and

implantation. These results indicate that, as marrow-isolated MSCs can be cultured successfully on a scaffold, they could be used for the tissue engineering of bone and implanted into patients to repair bone defects.

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Introduction: Mesenchymal Stem Cells for Tissue Engineering Bone

1.1 Background to thesis

The aim of this thesis is to investigate the use of cells isolated from human bone marrow for the healing of bone defects. It has been suggested that bone marrow contains cells that have the potential to differentiate into osteoblasts. Current treatment of bone defects that are too large to heal by primary intention require bridging by a structural support, which involves the use of metal prostheses and often additional bone graft to encourage bone union, neither of which are ideal.

Tissue engineering is the study of generating tissue from cells cultured in the laboratory to repair damaged tissues that would not heal without intervention. Engineering of tissue grafts requires the use of appropriate scaffolds on which to grow 3-dimensional (3-d) cellular structures and which aid the transfer of this tissue to the site of defect. Therefore, **the hypothesis investigated in my thesis was that cells isolated from human bone marrow could be stimulated to differentiate into osteoblasts and that these cells, when cultured on a scaffold could be used in the tissue engineering of bone, as the constructs could potentially be implanted into patients' bone defects resulting in increased healing.**

1.2 Clinical conditions requiring bony reconstruction

There are many causes of tissue loss, through for example, trauma, infection, neoplasm and metabolic disorders, as well as genetic and congenital conditions, and there are as many different tissue types from which loss can occur. As this is such a widespread problem that is difficult to treat, the cost to society is vast. Since disability can result from tissue loss, the goal is healing with the restoration of function.

Examples of orthopaedic situations that result in bone loss, which will not heal, include bone defects associated with fractures, bone tumour excision, revision arthroplasties and spinal fusion surgery, for which interventional treatment is therefore required to prevent loss of function. Treatment options depend on the tissue type, size and site of defect, and include autologous grafting, allograft implantation, synthetic substitutes or the use of metal prostheses. However, each of these treatments has limitations (see 1.3 Current bone defect treatments).

1.2.1 Fractures

Fractures usually heal by primary union. However fracture non-union, defined as a fracture that has failed to unite 6 months after injury, do occur. A fracture will not repair if there is a gap between the fracture ends that is too large, either due to bone loss, separation by tissue or distraction, all of which are more common following open fractures. In these situations, where primary healing has not occurred, other methods of repair are needed.

The concept of a critical bone defect is the smallest sized bony gap that will not unite spontaneously (Schmitz & Hollinger 1986). It has been suggested that a distance greater than the diameter of the bone is the critical defect size. This critical defect concept has been used experimentally in animals to test healing of segmental bone defects.

1.2.2 Bone tumours

Bone tumours are another source of bone tissue defects. Primary bone tumours are rare, especially when compared to metastatic deposits. However, metastases in bone are usually multiple and therefore treatment involves the maintenance of structural skeletal function by prevention or treatment of pathological fractures, rather than curative procedures. Breast, prostate, renal, thyroid and lung carcinomas are the most frequently found metastases in the bone.

Primary bone tumours can be classified into benign and malignant tumours. Osteoid osteoma are the most common benign tumours, others include osteoblastomas, osteomas and osteochondromas. The need for excision of these tumours varies with each case depending on certainty of diagnosis, possibility of malignant transformation, site and size of lesion.

Osteosarcoma is a malignant bone tumour, where proliferating spindle cells produce neoplastic stroma. They tend to occur in adolescents and affect males more than females. Fifty percent of osteosarcomas occur in the proximal tibia or the distal femur, in the metaphysis of the bone. Osteosarcomas can be divided further into different

types of osteosarcoma with varying degrees of malignancy. These tumours invade the bone locally, but also metastasise. Historically treatment involved amputation.

Recent use of adjuvant chemotherapy with methotrexate, doxorubicin, cisplatin and adrimycin has been shown to improve survival of osteosarcomas from about 15% to nearer 50% (Bacci et al. 1991). Advances in imaging such as MRI and CT over the last three decades, have allowed the margin of tumours to be more accurately defined. These two improvements have made it easier to excise malignant bone tumours rather than amputating. Subsequently, studies have shown that limb salvage does not shorten the disease free interval or reduce the long-term survival compared with amputation (Simon et al. 1986; Springfield et al. 1988), hence modern treatment aims towards limb salvage.

The result of tumour excision and limb salvage is a bony defect that needs to be bridged to maintain skeletal function. At the present time this is achieved using autologous and allogenic bone graft, autologous vascularised fibula graft or massive prosthetic implants.

1.2.3 Joint fusions

Spinal fusion is a surgical procedure whereby sequential vertebrae are fused together. This can be performed to relieve pain at the joint, by preventing movement between the vertebral bodies. Over 20,000 lumbar spinal fusions are performed in the US every year. Indications for lumbar spinal fusion include traumatic spinal injury, weakness due to infection or tumour, deformities such as scoliosis or spondylolisthesis, after discectomy for degenerative disease, and in association with decompression for spinal stenosis (Esses & Huler 1992).

Bone graft is used between the vertebrae to fuse them together and it is the graft that once united, provides structural strength rather than the metal fixation, which is used initially to hold the position of the spine. Currently, although autologous bone graft is taken from the patient's posterior iliac crest at the time of surgery, this is often insufficient and is therefore mixed with allograft, to increase the volume. Furthermore, there is a significant incidence of donor site morbidity associated with autologous bone

graft harvest (Laurie et al. 1984) and allograft also has limitations (see 1.3 Current bone defect treatments).

1.2.4 Revision arthroplasty

Total hip arthroplasty is one of the most successful treatments for osteoarthritis of the hip as it eliminates pain, thereby improving quality of life. In the UK 50,000 total hip replacements are performed each year, of which 9% are revision procedures. The Swedish hip register quotes that 71% of revision arthroplasties were due to aseptic loosening, the most common reason for revision surgery. Furthermore, a long-term study has shown that the probability of aseptic loosening after 20 years was 73% for a Charnley hip replacement (Jacobsson et al. 1996). Osteolysis induced by wear particles has been implicated as a cause of aseptic loosening, as it results in bony defects around the prosthesis both in the femur and acetabulum (Horowitz et al. 1993; Jiranek et al. 1993). As patients live longer and hip replacements are performed on younger patients, the number of revision operations will increase.

Bone defects that result from aseptic loosening need to be filled at the time of revision surgery, to ensure fixation of the new prosthesis. Currently, these defects are usually filled using a mixture of autologous bone graft, allograft and cement. However, the success of bone grafting has been shown to vary in the treatment of such bone defects (Gerber & Harris 1986) and custom-made prostheses are often required as graft materials are not sufficient to fill the defects.

1.2.5 Other causes

There are also other causes of bone lesions, which may manifest themselves as segmental bone defects. Osteomyelitis, especially when an infected sequestration is present, leads to the formation of bone defects and other conditions, such as hyperparathyroidism and gout, can present with lytic lesions in bone.

1.3 Current bone defect treatments

1.3.1 Bone grafts

Osteogenesis, in relation to bone grafts, refers to the presence of cellular elements within a graft which once implanted synthesise new bone. Osteoinduction infers that the graft has properties that recruit and stimulate mesenchymal stem cells (MSCs) within the host tissue to differentiate into osteoblasts. The term osteoconduction refers to a scaffold structure that facilitates the ingrowth of bone.

1.3.2 Autologous tissue graft

Autologous grafting is where tissue is taken from another site in the body and transplanted into a defect. In the case of bone, graft can be taken from the patients' iliac crest. The advantages of autologous grafting are that there is no risk of immune rejection and that the graft contains the patient's own osteoprogenitor cells and osteoinductive factors, which stimulate the formation of bone following implantation. Furthermore, as this type of graft is taken at the time of surgery, under sterile theatre conditions, the risk of pathogenic contamination and transmission of infection on the graft is reduced.

However, harvesting of bone graft can result in donor site morbidity, which manifests itself as pain and scarring. Additional risks at the donor site include infection and haematoma formation. The incidence of donor site morbidity, following bone graft harvesting from the iliac crest, was 72 out of 104 harvests (Laurie et al. 1984). The amount of graft available for grafting is limited to 10 – 15ml from the anterior iliac crest (Wheeless 1996) and this is often insufficient.

1.3.3 Allogenic bone graft

Allogenic graft is tissue that is taken from one person and transplanted into another. In the case of bone allograft, the main supply comes from femoral heads donated by patients, following total hip arthroplasty. Use of freshly obtained allograft induces an immune reaction, resulting in likely graft rejection. The graft therefore, either has to be blood group and HLA matched, or processed to reduce the immune reaction. In comparison to organ transplantation, where it is not possible to remove all the antigens, bone graft can be processed to reduce this immune reaction. The aim of this

processing is to reduce the immune response with minimal effect on the biomechanical strength and osteogenic properties of the graft.

In the UK, allograft is commonly washed to remove any marrow and blood, the fat is removed with alcohol and the graft is cleaned by ethylene oxide. Alone this is insufficient to produce an acceptable level of host immune response and hence, most grafts are also freeze-dried, which significantly reduces the immune response, but also kills osteogenic cells, with the result of reducing primary osteogenesis. The process also decreases the biomechanical strength of the graft (Wheeless 1996). Therefore, cancellous allograft only acts as an osteoconductive porous scaffold for the ingrowth of host bone.

Additionally, the use of allograft carries the risk of transmission of infection, both bacterial and viral. Bacterial contamination of femoral heads donated for use as allograft was found to be 22% in one study (Sommerville et al. 2000) and the risk of deep bacterial post-operative infection has been approximated to between 10 – 15% (Dick & Strauch 1994; Lord et al. 1988).

The risk of transmission of viral infections such as hepatitis B, C and HIV by allograft is greatly reduced if the graft is washed and freeze-dried, as this processing removes most of the marrow and blood, although it does not constitute sterilization. The donors are therefore screened at the time of donation and six months later for viruses that are transmitted via blood. The estimated risk of transplanting graft from an HIV positive patient has been calculated as less than 1 in 1.6 million (Buck et al. 1989) and there have been no reported cases in the US of viral transmission by allograft since 1995.

Although autoclaving bone graft sterilises it, reducing the risk of transmission of infection, heating also denatures proteins including Bone Morphogenic Proteins (BMPs). However, cleaning allograft with ethylene oxide and freeze-drying does not destroy all BMPs, thus some osteoinductive properties remain in the graft (Goldberg & Stevenson 1987).

Unfortunately, the availability of allograft is limited due to the lack of donors, each of whom has a fixed supply. Furthermore, the bone needs to be processed and stored in

tissue banks, which are expensive to run. Thus, the demand for allograft for use at revision hip surgery has been predicted to exceed supply in the next 5 years (Galea et al. 1998).

When comparing autologous bone graft with allograft, the most noticeable difference is that the incorporation of allograft into bone is slower, resulting in longer healing times (Glancy et al. 1991). This has been attributed to the immune response induced by allograft (Goldberg & Stevenson 1987).

1.3.4 Synthetic substitutes

Due to the limitations of autologous and allogenic bone grafts outlined above, especially the predicted shortfall in supply, much research has been carried out into potential synthetic materials. The ideal bone graft substitute would be biocompatible, absorbable, osteoconductive, osteoinductive and structurally similar to bone. Various synthetic bone graft substitutes are now available.

Current grafts, commercially available, can be categorised depending on the material used: demineralised bone matrix (DBM), DBM mixed with a second substance, calcium phosphate based materials and calcium sulphate based materials.

DBM is formed from bone graft that has been decalcified by treatment in hydrochloric acid, leaving the bone-protein matrix. There is evidence to suggest that segmental bone defects grafted with plasma coated DBM heal with a similar torsional strength as autologous bone grafting (Bolander & Balian 1986). However, DBM is subject to similar risks and limitations as allograft and lacks the structural strength of allograft.

DBM has also been mixed with substances, such as glycerol, to make a pliable material that can be injected as a gel, i.e. Grafton® (Feighan et al. 1995) and with calcium sulphate to form putty that can be used to fill irregular defects, i.e. Allomatrix®. Although these mixtures may have improved structural properties, they still require an allograft substrate.

Calcium phosphate, as one of the main components of bone mineral, has been used in various forms as bone graft substitutes. Hydroxyapatite (HA) has been derived from coral to make grafts that are porous in structure resembling trabecular bone – the resulting grafts can be packed into a bone defect. In an animal study, 48 months after implantation, autologous graft contained 73% bone, whereas the HA contained 51% bone (Holmes et al. 1987). Thus, although HA is poorly absorbed, it is osteoconductive, but additionally, it is fragile to handle.

Mouldable hydroxyapatites have been developed to improve the handling properties of the HA. An example of this is Orthofix®, which is made from tetracalcium phosphate and dicalcium dihydrate and is in a form similar to cement. However, the disadvantage of this viscous substance is that it can be difficult to squeeze into small bone defects.

Lastly, calcium sulphate has been used, as it is a biologically inert material that can act as a scaffold for the ingrowth of bone. It is available in the form of pellets, known as Osteoset®, which dissolve rapidly, within a couple of months of implantation. Although a disadvantage of HA is that it has been suggested to be absorbed too slowly, in comparison calcium sulphate based materials may fail before the bone has healed. Therefore none of these synthetic bone graft substitutes are ideal.

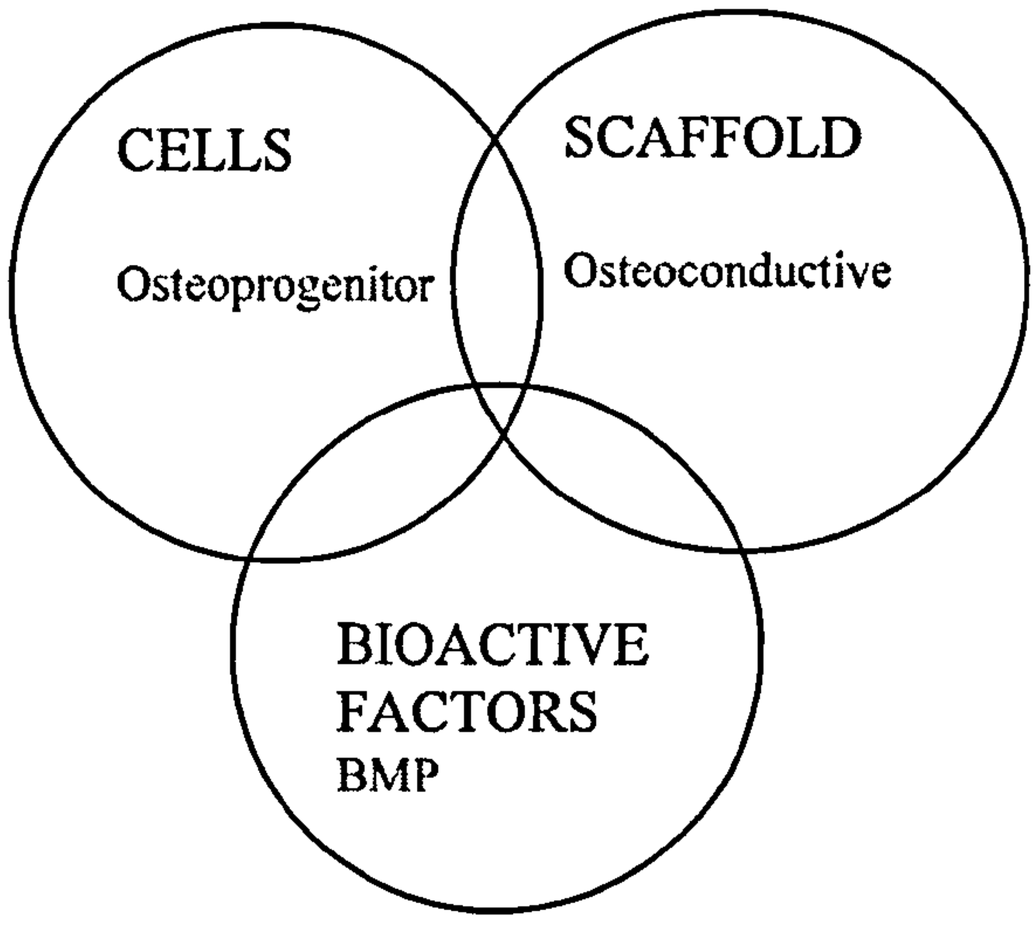
1.4 Tissue engineering

The definition of tissue is a group of cells united to perform the same specific function. Tissue engineering uses scientific methods of biology, chemistry and engineering to grow and maintain living tissue for regeneration (Langer & Vacanti 1993; Oreffo & Triffitt 1999). These techniques have been applied to repair damaged liver, pancreatic tissue in the case of diabetes mellitus and for the creation of blood vessels (Martin et al. 1997). However, for the purpose of my thesis, the use of tissue engineering of bone will be discussed.

For the tissue engineering of bone, the aim is to generate tissue from cells on a structural scaffold *in vitro* for implantation *in vivo* to repair defects. One of the significant benefits of tissue-engineered material over the use of allograft is that autologous cells can be used. In comparison to autologous tissue grafting, tissue

engineering requires a smaller donor site, as harvested cells can be expanded in culture, reducing donor site morbidity.

1.4.1 Basic Components of Skeletal tissue engineering



It is suggested that three factors are necessary for skeletal tissue engineering: cells, scaffold and bioactive factors.

1.4.1.1 Cells

Progenitor cells would be useful for tissue engineering due to their capacity for proliferation. However, they need to be stimulated to differentiate down the specific lineage and synthesise extracellular proteins, forming a matrix. Bone marrow is a source of osteoblast progenitor cells (see 1.7 Mesenchymal stem cells), thus isolation of these cells will be investigated for tissue engineering in this thesis.

1.4.1.2 Scaffold

For tissue engineering the scaffold used needs to meet certain criteria. Firstly, the scaffold is required to support the growth of cells, therefore the surface material must be biocompatible allowing cell attachment. The scaffold should also be porous, to encourage the diffusion of nutrients and colonisation of cells into its centre in a uniform distribution. Once implanted, a porous scaffold will also allow rapid vascularisation and the material should be absorbed as cellular regeneration occurs.

1.4.1.3 Bioactive factors

Osteoinductive agents used in tissue engineering are chemotactic for osteoprogenitor cells. These agents include growth factors such as fibroblastic growth factor, insulin growth factor, platelet-derived growth factor and in the case of bone, BMPs. This last factor has been used with a combination of HA scaffolds and been shown to improve repair of bone defects in rats compared with HA alone (Takagi & Urist 1982).

However, the dose of BMP necessary to induce bone regeneration is many times greater than those detected in normal bone tissue (Boyne et al. 1997; Howell et al. 2002; Noshi et al. 2000). Large doses of bovine BMP would cause concern in human clinical trials and recombinant BMP is expensive. It may be possible to use gene therapy to enhance cellular production of these factors, as it has been shown that MSCs transfected to encode for BMP-2 increase healing of large bone defects (Lieberman et al. 1998).

1.5 What is a stem cell

Stem cells have been the subject of much research over recent years due to their potential for medical treatments. The definition of a stem cell however, has remained contentious. Stem cells have been described as "cells with the capacity for unlimited or prolonged self-renewal that can produce at least one type of highly differentiated descendant" (Watt & Hogan 2000). A further definition of stem cells is "a cell type by which in the organism can maintain its own numbers, in spite of physiological or artificial removal of cells from the population" (Lajtha 1982).

A key factor is that all the different cell types present within a foetus are derived originally from a single cell. Thereby, cells present early within the embryo must be able to differentiate into any cell type and are thus known as totipotent stem cells. As these totipotent cells are usually derived from embryos of aborted foetus or *in vitro* fertilised eggs, ethical considerations have restricted embryonic stem cell research (Annas et al. 1996).

It has been shown that such cells, with the capacity to differentiate into different cell types, can be derived from various adult mammalian tissues and these cells have been

shown to possess some of the qualities of stem cells. However, in addition to the controversy on the definition of stem cells, no specific marker has been found to identify all stem cells, making the labelling of stem cells within a tissue difficult.

Another key factor is that, in contrast to embryological development, the role of cell division in the adult is to maintain a constant number of each cell type within tissue. Human tissue can be classified into three types denoted by the rate of cell turnover: static cell populations, conditional renewal populations and permanently renewing populations (Leblond 1963). Each tissue type has been found to contain cells that meet the criteria for stem cells, thus it is suggested that these cells are the source of new cells.

The first type of tissue is composed a static population of cells, which do not regenerate in the adult and most of the development is antenatal, for example nerves. However, within the central nervous tissue of rats and mice, there are cells with the capacity for self-renewal and differentiation, hence they fulfil the current definition for stem cells (Doetsch et al. 1999; Johansson et al. 1999). This suggests that other factors as well as the presence of these cells are necessary for regeneration.

Liver is an example of the second tissue type identified, as cell division is in response to stimuli, allowing renewal of the tissue. Hepatic stem cells have been found to persist in liver as a source of progenitor cells that can differentiate to replace mature hepatocytes (Sell 1990).

Lastly, there are tissues comprising of mature cells with short half-lives that are constantly replaced by proliferating progenitor cells, for examples blood and skin. Within the haematopoietic lineage, a common precursor cell has been identified by the CD34 antigen (Dexter & Spooncer 1987). This and other antigens have aided characterisation of the lineage, showing that a multipotent stem cell (cells that can differentiate down several cell lineages within a tissue) is present, which differentiates into different types of blood cell (Baumheter et al. 1993; Huss et al. 2000).

Furthermore, embryonic stem cells can be stimulated to differentiate into cells from different types of tissues, suggesting potency between different lineages. There is

evidence that some stem cells from adult tissue may possess this property, as marrow stem cells can be stimulated to differentiate into hepatic cells (Petersen et al. 1999) and neural stem cells differentiate into haematopoietic cells (Bjornson et al. 1999).

In summary, the main properties of stem cells are their ability to maintain a stem cell pool and to produce various differentiated cells.

1.5.1 Self-renewal

Stem cells are thought to divide to produce a progenitor cell, which may be committed to a specific cell type, and a daughter stem cell. These progenitor cells multiply rapidly before dividing into differentiated cells, thereby increasing the number of differentiated cells produced from a single stem cell. Therefore, although stem cells are present at a low density within the tissue and divide infrequently, the number of differentiated cells can be maintained by the pool of progenitor cells that is created from the division of a single stem cell (Morrison & Weissman 1994; Watt & Hogan 2000).

1.5.2 Methods of Division – asymmetric & symmetric

The division of stem cells to produce a differentiated cell and a stem cell can be achieved by two methods of division. Asymmetric division, where a stem cell divides into a stem daughter cell and a differentiated daughter cell, and symmetric division, where a stem cell divides into either two stem or two differentiated cells. On average, both methods achieve equal numbers of stem and differentiated cells per population, hence the method of division is indistinguishable from examination of tissue. Asymmetric division is observed in unicellular organisms and invertebrates such as the Drosophilia ovary and symmetric division is more frequently seen in mammalian cells (Morrison et al. 1997).

1.5.3 Control of division

Stem cell proliferation can be a normal response to tissue damage, whereby cells divide and produce differentiated cells to replace cells (Forge et al. 1993). In this case, cell division could be stimulated by mitogens released by cell death or the removal of a

protein normally produced by healthy cells that acts in a negative feedback mechanism.

In addition to this, the division of stem cells into a differentiated cell and a replacement stem cell is controlled by intrinsic and extrinsic factors. The intrinsic mechanisms for asymmetric divisions involve nuclear factors controlling gene expression and chromosomal modifications.

Extrinsic, environmental factors have been identified in the control of mammalian stem cell division. The idea of a niche model was developed, suggesting that stem cells reside within a specific microenvironment that keeps them in their stem cell state (Hall & Watt 1989). Therefore, when a stem cell divides there is only limited space within this area for the presence of one stem cell, for self-renewal. The other daughter cell leaves the niche, subsequently differentiating. Thus, as the local environment affects the phenotype of the daughter cells produced by symmetric division, this can limit the proportion of stem to differentiated cells (Kimble et al. 1992; Potten & Loeffler 1990).

The surfaces of keratinocyte stem cells express β1 integrin, the adhesion of which to the extracellular matrix, preventing its loss has been found to suppress differentiation (Jones & Watt 1993). Thus, while this integrin is expressed, cells are held within the stem cell niche thereby preventing differentiation. Following loss of this protein, cells leave the niche, where extracellular matrix proteins can then act as extrinsic factors to stimulate differentiation.

Further external control of stem cell division within the niche is achieved through cell-cell contact via the Notch receptor. The Notch family are transmembrane receptors that are involved in signalling pathways between cells directing them to adopt different cell fates. Notch activity is required for the differentiation of sensory precursor cells in the Drosophilia eye (Artavanis-Tsakonas et al. 1999).

1.5.4 Control of differentiation

In addition to the environmental factors discussed above, differentiation occurs as a result of cells exiting the stem cell state and entering a specific lineage. There is

evidence that entry into a differentiated cell lineage is controlled by gene expression. PIE-1 is a germ cell specific nuclear protein that has been identified as an inhibitor of the expression of genes involved in the commitment of cells to a differentiated state. Therefore, the presence of this protein prevents differentiation of the cell. PIE-1 is inherited by only one daughter stem cell during asymmetric stem cell division due to blastomere distribution (Mello et al. 1996). Thereby the presence of PIE-1 in one of the cells maintains its stem cell state, but its lack in the other cell allows differentiation.

Further investigation into the choice of differentiation lineage by multipotent stem cells suggests that growth factors and cell-cell interaction influence the outcome (Shah et al. 1996). However, further research is needed into the methods that control stem cell division and differentiation, as few controlling factors have been identified so far.

1.6 Stroma

Bone marrow connective tissue network is called stroma, the word derived from Greek for a bed, as the function of stroma is to support bone marrow by providing a structural environment for haematopoiesis. Stroma involves many types of cells and forms a complex extracellular matrix, which supports the haematopoietic process. Haematopoietic precursors express CD34 (Clark & Keating 1995), thus the presence of this protein on cell membranes has been used to differentiate between haematopoietic precursors and fibroblastic stromal cells (Huss et al. 2000).

1.7 Mesenchymal stem cells

In the trilaminar embryo, the mesodermal (middle) layer forms between the ectodermal and endodermal cell layer. This mesodermal cell layer contains mesenchymal stem cells (MSCs), which develop into connective tissue (mesenchyme). MSCs are a population of multipotent mesenchymal progenitor or stem cells that persist after birth.

1.7.1 History and nomenclature of MSCs

Alexander Friedenstein was a Polish scientist working in Moscow, when he pioneered research into bone marrow and stromal precursor cells. He discovered evidence for

fibroblastic progenitor cells in bone marrow by showing that colonies, measuring about 0.5cm in diameter and containing thousands of fibroblastic cells, formed when bone marrow was plated *in vitro* for 10 days. He suggested that these colonies resulted from multiple divisions of a single cell and that this was characteristic of stem cells. Therefore, Friedenstein named these cells Colony Forming Units-Fibroblastic (CFU-F) (Friedenstein et al. 1966; Friedenstein et al. 1970).

Maureen Owen, working with Friedenstein and Beresford, adopted the term marrow stromal cell in the 1980's to describe cells that are isolated from bone marrow with the potential for skeletal differentiation (Beresford 1989; Owen & Friedenstein 1988). However, Triffitt raised concerns with the use of this term, as stromal cells also include reticular cells, epithelial cells and macrophages that are haematopoietic in origin and do not differentiate down skeletal cell lines (Seshi et al. 2000; Triffitt 1996). Caplan has consistently referred to these isolated marrow cells as mesenchymal stem cells (Caplan 1991), although the use of this term has been criticised as the stem cell nature has still to be fully proved.

1.8 Characteristics of MSCs

1.8.1 Self-renewal capacity

As discussed above, stem cells have the capacity for self-renewal, maintaining a population of stem cells within the tissue, despite differentiating into other cell types. It has been shown that in culture 20% of MSCs are in the quiescent G_0 phase of the cell cycle. Cells that are in this phase replicate infrequently and have been shown to possess the stem cell characteristic of self-renewal, hence this is consistent with the presence of a low density of MSCs in tissue (Conget & Minguell 1999).

When regeneration of tissue is needed following cell death, the stem cells have the ability to rapidly produce a large number of progenitor cells. This high proliferation capacity was first noted by Friedenstein (Friedenstein 1976) and has been confirmed more recently, where proliferated MSCs have been shown to retain their differentiation ability (Bruder et al. 1997b).

1.8.2 Bone marrow cells' differentiation

The ability of marrow to form bone was first suggested by Goujon as early as 1869. Little more was discovered about the potential of cells present in marrow until the last three decades, when it has been shown that they have the ability to differentiate down the different mesenchymal cell lines, including chondroblastic, adipocytic and osteoblastic. In 1970, Friedenstein first provided evidence that CFU-F were multipotent stromal cells capable of supporting haematopoiesis, as when mouse marrow CFU-F cells were transplanted under the renal capsule several cell lines – bone, adipose and marrow reticular tissue were found to produced (Friedenstein et al. 1970). Later, evidence of host cellular haematopoiesis within this heterotopic bone was found (Friedenstein 1980).

Bone marrow was initially shown to have osteogenic potential. When rat marrow was implanted *in vivo* in a diffusion chamber, about 40% of CFU-F formed osteogenic tissue within the chambers (Friedenstein 1976). More recently, these results have been confirmed, as when human marrow was loaded into a diffusion chamber and implanted intraperitoneally into athymic mice, mineralised tissue formed after 4 to 8 weeks (Bab et al. 1988b).

However, cartilage has also been noted to form in the centre of the chamber (Bab et al. 1984), suggesting that marrow contained both osteoblastic and chondrocytic precursors. The formation of cartilage in the centre of the chamber is consistent with chondrogenesis occurring preferentially in an avascular environment.

In an additional experiment, when HA ceramics have been loaded with bone marrow and implanted subcutaneously or intraperitoneally into rats, it has been shown that bone forms in the pores of the ceramic 3-4 weeks after implantation (Ohgushi et al. 1989a). This has been a reproducible finding that confirmed the presence of an osteoblast precursor cell in bone marrow.

1.8.3 MSC differentiation

MSCs that have been isolated from whole bone marrow have been proposed as skeletal precursors, as discussed. Evidence for MSCs possessing the ability to differentiate into

osteoblasts was demonstrated in studies where MSCs were separated from rat marrow, grown *in vitro*, then implanted subcutaneously on HA scaffolds. Two weeks after implantation bone was noted to have formed, confirming that isolated MSCs from marrow differentiate into osteoblasts (Dennis et al. 1992; Goshima et al. 1991b). This finding has been confirmed by various groups, showing that human MSCs loaded onto HA and implanted subcutaneously in an animal model form bone, thus this method has also been used as an assay for the presence of MSCs (Haynesworth et al. 1992b; Lazarus et al. 1997).

In vitro work has further characterised the differentiation potential of MSCs, by showing that marrow-isolated cells, with homogenous morphology, do not differentiate in standard culture conditions (Beresford 1989), but can be stimulated to differentiate into osteoblasts, chondrocytes and adipocytes under appropriate conditions. Various research groups have shown that MSCs isolated from rat marrow can be differentiated into osteoblastic expressing cells when cultured *in vitro* using β-glycerophosphate, ascorbic acid and dexamethasone (Maniatopoulos et al. 1988; Ohgushi et al. 1996a). When cultured under the same conditions, human MSCs also differentiate into osteoblastic cells, identified by the increased production of bone markers (Jaiswal et al. 1997). Therefore, marrow-derived MSCs possess osteoblastic potential, although this may be via an intermediate osteoblastic progenitor cell.

In addition to MSCs possessing osteogenic differentiation potential, the multipotency of these marrow cells has been suggested, as MSCs have been differentiated into chondrocytes. Conditions that stimulate chondrocytic differentiation of rat MSCs in culture include dexamethasone, TGF-β1 and 3-dimensional cell aggregates (Johnstone et al. 1998). These culture conditions have also been shown to have the same effect on MSCs isolated from human marrow (Mackay et al. 1998).

Human MSCs can also be differentiated into adipocytes, following culture with larger doses of dexamethasone (Cui et al. 1997). The results of these experiments show that MSCs isolated from human bone marrow can be differentiated down the osteoblastic, chondrocytic and adipocytic cell lineages, which confirms their multipotency and is

further evidence of their mesenchymal stem cell origin (Pittenger et al. 1999). These MSCs are the source of cells responsible for connective tissue healing.

Of the lineages, the osteoblastic and adipocytic cell lines seem to be more closely related, as there is evidence that a single transduction pathway controls the differentiation between these two lineages. The levels of MAP kinase increase when the cells are stimulated to differentiate into osteoblasts. Conversely, the blockage of this pathway causes the cells to differentiate into adipocytes (Jaiswal et al. 2000). This suggests that MSCs differentiated into osteoblasts or adipocytes depending on the MAP kinase levels. It is of interest to note that MSCs sub-cultured many times retain the potential to differentiate into osteoblasts, but fail to differentiate into adipocytes after the third passage (Conget & Minguell 1999).

There is histological evidence from studying the embryonic development of chick limbs that, during endochondral ossification, the cartilaginous framework is replaced by bone, but chondrocytes do not differentiate into osteoblasts (Pechak et al. 1986). Caplan and Bruder have attempted to outline the osteoblastic differentiation lineage using embryological development as a model (Bruder et al. 1994b).

1.8.4 Homogeneous or Heterogeneous Population

There has been much debate as to whether MSCs isolated from bone marrow are a homogeneous or heterogeneous population of cells. Certainly, marrow-derived adherent fibroblastic cells have been referred to as MSCs by several groups of investigators, thus assuming the status of homogeneous population. For example, after isolation of adherent cells and negative immunoselection for antigens known to be specific for haematological and epithelial cells, the cells were referred to as a homogeneous population of MSCs (Rickard et al. 1996). Furthermore, MSCs express a homogeneous set of antigens suggesting a single cell type, although the individual antigens are not unique to MSCs being shared with epithelial cells (Conget & Minguell 1999).

Cell culture work has raised concerns about the homogeneity of MSCs. Owen's experiments proposed that cells within colonies were heterogeneous, as the cellular expression of alkaline phosphatase (ALP) varied between cells throughout a colony

(Beresford & Owen 1998). These findings could also be explained by an initial stem cell dividing into a daughter stem cells and a progenitor cell, which in turn differentiates into an ALP-producing osteoblast. Thus, an isolated population of homogenous MSCs would produce a heterogeneous population of cells, although these observations would be indistinguishable from an initially heterogeneous population of cells.

Further, as isolated MSCs are low in density and proliferate rapidly, it is difficult to study the initially isolated population. However, comparison between marrow cells from different donors and collection at different times has revealed large variations in growth rate and osteoblastic differentiation potential (Phinney et al. 1999).

It has also been shown that only 58% of MSCs when assayed *in vivo* have the capacity to form bone (Kuznetsov et al. 1997). This may be the result of stem cell renewal to maintain a pool or progenitors committed to other cell lineages. This suggests that mesenchymal cells isolated from marrow form a heterogeneous population of cells, but until there are specific markers for stem cells and each precursor cell, it is likely that this question will remain unanswered.

1.9 Markers of MSCs

In the haematopoietic cell lineage, the multipotential stem cell, committed progenitor cells and mature cells were identified initially by their morphology (Dexter & Spooncer 1987). Furthermore, markers for specific colony-stimulating factors that stimulate the differentiation of cells at each step along the lineage have been identified, making it possible to characterise cells at each step (Orlic & Bodine 1994). However, the characterization of a MSC and its derivatives has proved more difficult due to the lack of identification of similar factors. The nature of stem cells to differentiate into multiple cell lines means that the phenotype varies and specific markers for cells at different stages have yet to be identified.

Despite these difficulties, various groups have investigated ways to identify markers for MSCs. Simmons identified a monoclonal antibody Stro-1 that recognizes marrow stromal progenitor cells in bone marrow (Simmons & Torok-Storb 1991). Stro-1

antibody binds to 10% of mononuclear cells in bone marrow, but was not found to react with haematopoietic precursors in the marrow (Simmons & Torok-Storb 1991). Stro-1 separated marrow cells were shown to differentiate into osteoblasts, chondrocytes and adipocytes when cultured with the correct stimuli (Ahdjoudj et al. 2001). Furthermore, MSCs have been shown to express high copy numbers of Stro-1 and therefore, Stro-1 has been suggested as a useful marker of MSCs.

Hybridomas can also be used to raise monoclonal antibodies against specific cellular antigens. Caplan and colleagues have generated hybridoma lines that secrete antibodies that recognise antigens on the cell surface of differentiating isolated marrow cells, but do not label mature osteoblasts. Despite the identification of these monoclonal antibodies no exclusively specific marker for MSCs has been developed.

1.10 Markers of osteoblastic lineage cells

Characterisation and identification of cell markers for osteoblastic cells at each stage of differentiation has been researched, partly to aid characterisation of MSCs, as well as defining the osteoblastic cell lineage.

Attempts have been made to raise monoclonal antibodies against cells along the osteoblastic lineage and monoclonal antibodies SH-1 (Bruder & Caplan 1990), 2, 3 & 4 (Haynesworth et al. 1992a) have been raised, with varying combinations of expression being evident by cells at different stages of mesenchymal differentiation. The monoclonal antibody SB-10 recognises a further cell surface antigen expressed on isolated human marrow cells, but does not react with cells that express ALP, hence it is a marker of early osteoblast precursors rather than osteoblasts themselves (Bruder et al. 1997a). The expression of these antigens has been used by the authors as evidence for an osteoblastic differentiation pathway (Bruder et al. 1994a; Caplan 1991).

There are currently no other methods to confirm these stages of cell differentiation. Additionally, there has been limited success in confirmation of such stages, as these antibodies have reacted diversely to the cell surface, cytoplasm and extracellular matrix proteins, and very few antibodies have detected cells other than the most mature in the osteoblast cell lineage (Aubin & Turksen 1996).

Therefore, in addition to cellular markers, a further method of identification of cells at different stages of maturity is by the changing biochemical features of the cells. Extensive investigation of osteoblast progenitor cells by Aubin et al has suggested that the gene expression indeed changes as the cells mature from osteoblast progenitors to pre-osteoblasts to osteoblasts, and correspond with decreasing proliferation and increasing differentiation. Therefore protein or mRNA expression can be used as temporal differentiation markers. Using relative gene expression over time the three phases of osteoblastic differentiation postulated (Lian & Stein 1992), are:

1. Proliferation
2. Extra-cellular matrix maturation
3. Mineralisation

Markers of proliferation include the gene expression of histones, proto-oncogenes (such as c-fos and c-myc) (McCabe et al. 1995). From the study of these it has been suggested that osteoblast progenitor cells divide eight times before they differentiate and start to produce osteoblastic extracellular matrix.

Using methods to detect mRNA (poly-A-PCR and in situ hybridization) and immunocytochemistry to label individual cells, specific proteins were found to be modulated during the process of differentiation (Aubin 1998).

Initially active cells, during the proliferation stage, express collagen type 1, osteopontin and fibronectin. As the proliferation rate decreases, this is coupled with an increase in matrix production, with production of ALP and a second peak in osteopontin. This is followed by an increase in bone sialoprotein (BSP), a glycoprotein essentially found in mineralising connective tissue, and osteocalcin with the onset of mineralisation (Owen et al. 1991).

There are thought to be restriction points in the gene expression at the end of the first and second stage of osteoblastic differentiation. The extra-cellular matrix deposition contributes to both the reduction in proliferation rate and the development of the osteogenic phenotype. These interactions control whether cells proliferate further or

differentiate and are especially important in tissue remodelling and new bone formation (Stein et al. 1990).

However, there are variations in the stage of osteoblastic differentiation at which maximal levels of individual gene expression have been detected. For example, instead of peaking during proliferation, collagen type I mRNA was found to peak during matrix mineralisation (Malaval et al. 1994). This may reflect the method used to measure mRNA. However, there is evidence that the effect of growth factors, in stimulation of gene expression by the osteoblastic target cell, is affected by the individual cell's stage of differentiation. Therefore, this variation noted in gene expression, may result from different cell conditioning and may further be explained be the heterogeneity of MSCs at different stages of differentiation in culture (Liu et al. 1997), which, as markers of differentiation makes the use of gene expression by cells limited.

Osteoblast progenitor cells isolated from bone marrow can be separated on the basis of their expression of ALP. The cells that were found to be ALP positive formed bone nodules in culture without dexamethasone, however cells that did not express ALP needed the stimulation of dexamethasone to mature forming bone nodules (Turksen & Aubin 1991). It was therefore suggested these represent osteoblast progenitor cells in different stages of differentiation. However, it could also represent committed and uncommitted osteoblast progenitor cells, with the latter requiring further stimulation to trigger osteoblastic differentiation.

The characterisation of the postulated MSC cell line has therefore developed, initially from the identification and variation in different mature and precursor cell types.

1.11 Osteoblastic cell markers

1.11.1 Extracellular matrix proteins

Extracellular matrix proteins form the basis for bone synthesis, thus production of these proteins is characteristic of the osteoblastic phenotype and can be used as markers for osteoblastic cells *in vitro*. The following are a list of such proteins, the presence of which is used to confirm production of extracellular matrix:

Collagen type I is the single most abundant protein in the body and the major organic component of bone. It is produced during the proliferation phase of osteoblastic development, making it an early recognisable marker of osteoblastic function (Risteli & Risteli 1993). As seen under transmission electron microscopy, the collagen fibrils are arranged staggered end to end in an ordered cross-banding pattern.

Osteocalcin is an important non-collagenous protein of bone that is vitamin K dependent and has a low molecular weight (Brozovic 1976). It is synthesised exclusively by osteoblasts and ondoblasts (Price 1985), being produced during the mineralisation stage of osteoblastic differentiation. Osteocalcin is known to bind calcium mineral in bone, although its precise function is not fully understood. It may also act as a cytokine for the chemo-attraction of osteoblasts and osteoclasts (Malone et al. 1982). Consequently, osteocalcin levels in serum and synovial fluid have been shown to correlate with new bone formation and conditions of increased bone turnover, for example Paget's disease (Eyre 1997).

Osteopontin protein is located at the cell-matrix interface in mineralised tissue deposited by osteoblasts. Osteopontin binds to calcium within the extracellular bone matrix, anchoring osteocalcin in the mineral. The expression of osteopontin by osteoblasts is increased as a result of the over expression of Cbfa-1 (Tsuji et al. 1998). Additionally, mechanical forces have also been shown to influence osteopontin expression, as intermittent hydrostatic compression of osteoblasts in culture increases osteopontin and ALP protein and mRNA levels (Klein-Nulend et al. 1997; Kubota et al. 1993). It has been suggested that these forces act on cell attachment, modifying the gene expression via a signalling pathway.

Osteopontin is therefore a useful marker of osteoblastic activity, although it is a non-specific marker, as it is widely expressed in body fluids particularly at sites of inflammation. Evidence of this is seen in the elevated levels of osteopontin found in humans during metastatic cancer and sepsis. The expression of osteopontin in these conditions may result from the high number of proliferating cells.

Osteonectin is another protein present in the extracellular matrix of bone. Its action involves modifying the interaction between cells and the extracellular matrix (Termine et al. 1981). It has also been shown to increase the production of other proteins, including collagen, by fibroblasts and endothelial cells, therefore it a non-specific marker of osteoblasts. Furthermore, osteonectin mRNA expression was not found to change significantly during osteoblast development (Malaval et al. 1994), suggesting that it is less useful as a marker of osteoblastic cells.

Bone sialoprotein (BSP) is an attachment protein that is found exclusively in bone (Oldberg et al. 1988a). It acts as an integrin binding protein with an affinity for the vitronectin cell receptor and collagen, which suggests that it mediates the attachment between cells and collagen (Oldberg et al. 1988b). BSP has been identified, by immuno-histochemistry, preceding mineralisation of the extracellular matrix, suggesting that BSP is a necessary trigger for mineralisation (Roach 1994).

1.11.2 Isoenzymes

Alkaline phosphatase (ALP) is an enzyme that catalyses the hydrolysis of phosphate esters at an alkaline pH. Bone, liver and kidney isoenzymes of ALP have been identified and the skeletal isoform of ALP is a glycoprotein on the cell and nuclear membranes and in the cytoplasm of osteoblasts.

Gene expression for ALP begins, following the cessation of osteoblastic proliferation stage, increases during matrix maturation and declines with mineralisation. This implies that ALP is important during the initiation of mineralisation (Stein et al. 1990). Total ALP expression has been recognized as a reliable indictor of osteoblastic activity in the Saos-2 osteosarcoma cell line (Rodan et al. 1987) and ALP serum levels are frequently used clinically to assess both physiological and pathological bone turnover. Therefore, ALP is a widely used marker of osteoblast phenotype (Risteli & Risteli 1993).

1.11.3 Transcription factors

Signals initiate DNA transcription of the corresponding gene prior to synthesis of the extracellular proteins. Hence these signals, known as transcription factors, are present

within the cells before the protein and are indicators of early cell differentiation. **Cbfa-1** is a transcription factor within osteoblastic cells that is part of the runt-domain gene family and is essential for bone formation. It was found that Cbfa-1 knockout mice were unable to mineralise extracellular matrix to form bone, although osteoblastic cells expressed ALP. As a result the mice died just after birth due to suffocation (Komori et al. 1997).

Cbfa-1 expression has been detected in osteoblasts and T-cells, and weakly in chondrocytes and dermal fibroblasts, but it seems to be specific to connective tissue, as it is not found in fibroblasts elsewhere.

In the embryological development of mice, cells within mesenchymal condensations at 12.5 days post-conception expressed Cbfa-1 and by day 16 mesodermal cells destined to become skeletal tissue, in both intramembraneous and endochondral bone ossification centres also expressed Cbfa-1 (Ducy et al. 1997). This provides further evidence that Cbfa-1 is a marker of early skeletal development.

In rats, the DNA binding site for Cbfa-1 has been identified in the promoter region of the gene for osteocalcin (Merriman et al. 1995), suggesting that Cbfa-1 signals osteocalcin transcription. In addition to osteocalcin, in mouse mesenchymal cells osteopontin and BSP genes also have Cbfa-1 binding sites (Ducy et al. 1997). Therefore, the expression of these genes is also induced by the transient expression of Cbfa-1 transcription factor. As osteocalcin production in closely related to the mineralisation of bone, it has been suggested that Cbfa-1 is an essential factor for osteoblastic maturation, as suggested above. Therefore, it has been used as a marker for differentiating osteoblasts *in vitro* (Gori et al. 1999).

1.12 Potential clinical uses for MSCs

As discussed in section "1.2 Clinical conditions requiring bony reconstruction" there are many clinical conditions that are currently treated with various types of bone grafting. As these treatments are less than ideal, methods using bone marrow and MSCs could potentially improve treatment.

1.12.1 Evidence for the use of bone marrow to heal segmental bone defects

Red bone marrow has been known to form bone since the late 1800's, therefore it has been suggested that marrow could be used to augment bone healing. Marrow and marrow cells separated by centrifugation have been injected percutaneously into radial fracture defects, with the effect of increasing osteogenesis (Connolly et al. 1989b).

The Connolly group has since investigated the use of marrow in healing of 20 un-united tibial fractures with good results, suggested to be equivalent to autologous bone grafting. Nineteen of the 20 fractures, originally Gustilo-Anderson type III had failed to unite following multiple attempts however, after marrow injection 18 of the fractures united (Connolly et al. 1991b). This suggests that from within the marrow osteoprogenitor cells proliferated and differentiated to form bone.

The use of bone marrow to increase healing of a segmental bone defect has also been shown in rats, when porous calcium phosphate ceramics were loaded with marrow and implanted into the defect, bone union occurred after 2 months in 8 out of 12 defects (Ohgushi et al. 1989b).

1.12.2 Use of MSCs to heal segmental bone defects

As the ability of marrow to regenerate bone is attributed to MSCs and progenitor cells present within it, the use of MSCs to heal a segmental bone defect has been investigated. When culture expanded autologous MSCs were re-implanted on a ceramic into a canine bone defect, the healing rates improved after 16 weeks (Bruder et al. 1998a). Other groups have also improved healing of a critical bone defect in a sheep model, by implanting culture expanded MSCs on HA (Kon et al. 2000). However, the injection of isolated and culture expanded rat MSCs into a distraction model was not found to improve bone regeneration and healing, as new bone formed independently of whether cells were injected or not (Richards et al. 1999).

A myocutaneous flap can be used to transpose muscle and skin to reconstruct a tissue defect. As bone has been shown to form when human MSCs have been implanted on a HA scaffold on a latissimus dorsi flap in nude mice (Casabona et al., 1998), a myocutaneous flap could be converted to an osteo-myocutaneous flap. This procedure could be performed with autologous marrow and would avoid bone graft harvest and its complications.

1.12.3 Potential treatment of osteoporosis with MSCs

As osteoporosis causes at least 1.5 million fractures in the US per year, it is a key cause of morbidity and mortality in the elderly population. It is the result of an imbalance between bone re-absorption and production, due in part to reduced levels of protective oestrogen hormone, consequently reducing skeletal bone mass.

It has been suggested from *in vitro* research, that in animals the number of MSCs per volume of marrow decreases with age (Bergman et al. 1996; Huibregtse et al. 2000). It has also been suggested that the proliferation rate (Egrise et al. 1992) and differentiation rate of MSCs into osteoblasts is lower in osteoporosis (Rodriguez et al. 1999).

As noted above, the differentiation of MSCs down the osteoblastic and adipocytic cell lines is closely related (Jaiswal et al. 2000), (see 1.8.3 MSC differentiation section). In osteoporotic patients the decrease in bone volume correlates with an increase in adipose tissue (Gimble et al. 1996). Therefore, in osteoporotic bone the MSCs may be stimulated to differentiate into adipocytes instead of osteoblasts.

The potential for use of MSCs in the treatment of osteoporosis has already been investigated. Following intravenous injection of culture expanded MSCs into humans, engraftment into marrow may be as low as 1 – 2% (Caplan & Bruder 2001). Thus, for the treatment of generalised bone diseases, the cells need to be delivered on a carrier to the intended site or labelled with specific attachment proteins for tissue targeting.

1.12.4 Uses of MSCs in genetic bone disorders

Osteogenesis imperfecta (OI) is a genetic disorder (autosomal dominant), where osteoblasts produce defective type I collagen, which results in elastic connective tissue and weak bones that become deformed and fracture easily (Primorac et al. 2001).

Bone marrow has been used with success to treat 3 children with this condition (Gerson 1999). Allogenic, HLA matched marrow was infused intravenously into the patients. Three months after engraftment, bone biopsies showed that 1.5% – 2% of osteoblasts were of donor origin, resulting from infused mesenchymal progenitors migrating into the bone and giving rise to normal osteoblasts. However, even with this low cellular engraftment rate, the total mineral content of bone increased and the fracture rate was reduced (Horwitz et al. 1999).

An explanation for these observations is that in OI the severity of the disease is dependant on the proportion of normal to abnormal collagen produced. So the engrafted osteoblasts, although a small percentage of the total osteoblasts, may produce enough normal collagen to shift the balance resulting in clinical improvement. Although, further research is needed to determine the number of MSCs needed to give long lasting clinical improvement.

1.12.5 Possible use of MSCs in gene therapy

As MSCs are also known to disseminate through connective tissues when injected intravenously (Pereira et al. 1995), MSCs could have specific genes introduced into their genome by viral vectors. These cells could be engrafted into patients with connective tissue gene dysfunction resulting in the production of normal proteins, for example for the treatment of OI. This could also be potentially useful for delivery of genes to correct other diseases, such as the clotting disorders haemophilia (factor XIII deficiency) and Christmas disease (factor IX deficiency).

1.13 Aims and Hypothesis

In summary, current treatment of the common clinical problem of bone defects has limitations. As there is evidence that MSCs could be used to tissue engineer bone, my thesis will investigate the methodology and viability of this, to provide better treatment of such defects.

In order to establish a methodology for the tissue engineering of bone, various factors need to be first established. For instance it is necessary to show **that MSCs can be isolated from bone marrow aspirates harvested from patients.** Further, as MSCs are known to occur at low density in bone marrow, **the isolated cells would then need to be expanded in culture to produce the larger numbers** required for tissue engineering. It is also proposed **that bone marrow-isolated MSCs from every patient can be stimulated to differentiate into osteoblasts.**

For clinical use in bone defects, cells would require stability, afforded it is proposed by using a scaffold on which to grow a 3-d structure. As a major constituent of bone extracellular matrix, HA has been shown as osteoconductive and therefore a suitable material for the scaffold. HA has also been shown to encourage the osseointegration of bone when coated on metal prostheses (Furlong & Osborn 1991). **A key hypothesis of my thesis is therefore that MSCs will grow on HA and differentiate into osteoblasts without other stimulation in culture.**

The biological structure of engineered tissue used to replace lost tissue must be similar to the original. It is **hypothesised that when grown within a novel perfusion bioreactor, which simulates the environment of osteoblasts in bone, this will stimulate the osteoblastic differentiation of MSCs and the production extra cellular matrix.** As, to construct 3-d tissue a uniform distribution of cells is needed through the scaffold, it is also **hypothesised that the penetration of cells through a porous scaffold will be increased by culture in my bioreactor.**

Lastly, for these methods to be used clinically, it is necessary to devise a means of storing MSCs derived from marrow, without altering the osteoblastic potential of the

cells. **It is hypothesised that cryopreservation will allow the storage of freshly isolated MSCs without changing their cell function and potential.**

In conclusion, the general **hypothesis of the thesis is that a population of multipotent cells can be isolated from bone marrow and that the differentiation these MSCs into osteoblasts, chondrocytes and adipocytes can be controlled and maintained and that osteoblastic differentiation will be affected by surfaces on which they are cultured.** Further it is **hypothesised that growth and differentiation will increase in a 3-d bioreactor culture system and will not be affected by cryopreservation storage.** Therefore, my thesis will investigate the suitability of MSCs for the tissue engineering of bone, with the aim that this tissue could be implanted into patients' bone defects resulting in increased healing.

CHAPTER 2

Human Bone Marrow as a Source of Mesenchymal Stem Cells

2.1 INTRODUCTION

2.1.1 Background to chapter

The overall aim of this thesis was to investigate whether cells isolated from human bone marrow could be used in the tissue engineering of bone. Hypothetically, this engineered tissue could be used clinically as graft substitutes in the reconstruction of bone defects.

In this first chapter, several main factors need to be established. Firstly, that mesenchymal stem cells (MSCs) can be isolated from human bone marrow aspirates. Secondly, that such cells could be stimulated to differentiate into osteoblastic, adipocytic and chondrocytic cells in culture, thereby confirming the multipotency of the marrow-isolated mesenchymal cells. Furthermore, that the isolated cells can be expanded in culture to produce the large numbers of cells required for tissue engineering.

As the focus of this thesis is the tissue engineering of bone, in this chapter it was the potential of the isolated cells to differentiate into osteoblasts that was most closely studied.

2.1.2 Origins of the identification of Mesenchymal Stem Cells

As detailed in Chapter 1, in the late 1960s Friedenstein found that, when rat bone marrow was plated in culture colonies of fibroblast-like cells formed, which he called Fibroblastic-Colony Forming Units (CFU) (Friedenstein et al. 1970). The stem cell nature of these cells was suggested as, *in vitro*, large colonies of cells seemed to form rapidly from the initial division of a single cell. Several research groups have demonstrated the pluripotent nature of this cell type *in vitro* (Conget & Minguell 1999; Maniatopoulos et al. 1988; Pittenger et al. 1999), (see Chapter 1: MSC differentiation).

However, as previously discussed, a wide nomenclature has been applied to these cells in scientific literature (see Chapter 1: History and nomenclature of MSCs section). This variety is likely to have resulted not only from the heterogeneous nature of the

isolated cells, but also from the variation in cell phenotype expressed in different environments which may have resulted in the same cells being identified differently. Furthermore, lack of stem cell markers has limited the identification and labelling of marrow-isolated cells, also potentially resulting in different names being given to similar cells. In this chapter, the bone marrow-derived cells will be referred to as MSCs.

2.1.3 Distribution of MSCs in Bone Marrow

Studies outlined in Chapter 1 provide evidence that MSCs are present in bone marrow and have the potential for differentiation into cells of mesenchymal tissue. In adults, haematopoietically active marrow retracts to the medullary cavity of the axial skeleton, as can be seen by the colour of the marrow, which remains red in these bones compared with the yellow fatty marrow in the distal skeleton. Thereby, to obtain marrow that is rich in haematopoietic stem cells marrow is aspirated from the axial skeleton, for example the iliac crest or sternum. It is likely that MSCs will also be present in a higher frequency in this red marrow and bone marrow can easily be harvested from this site.

2.1.4 Source of MSCs

In addition to marrow, peripheral blood has also been investigated as a source from which MSCs are potentially recruited to sites of skeletal injury. Evidence for this has been documented by Huss et al, who found that peripheral blood contained fibroblastic cells with characteristics of MSCs, including the expression of CD34 and osteocalcin. Although in culture these cells initially expressed CD34, suggesting that they were haematological precursors (Huss et al. 2000), they lost the CD34 antigen during culture, which may be associated with the cells differentiating into MSCs. However, it should be noted that other researchers have not been able to isolate MSCs from peripheral blood (Lazarus et al. 1997). Although this does not exclude their presence, it would suggest that MSCs occur in too low a density in blood to be detected. Hence, for my study, bone marrow alone was investigated as a source of MSCs.

2.1.5 Isolation of MSCs from bone marrow aspirates

Bone marrow can be taken by aspiration from the medullary cavity of the posterior iliac crest of humans; this is routinely performed for the diagnosis of haematological conditions. The majority of low density MSCs have been found to be present in the initial part of the aspirate (Muschler et al. 1997). This has been suggested is due to the limited amount of marrow in the trabeculae local to the bevel of the needle, with further aspiration subsequent to this yielding venous blood only (Batinic et al. 1990). In my thesis, 2ml samples of human bone marrow were obtained by aspiration from the iliac crest of patients following local ethical committee approval and informed consent.

Various methods have been investigated for the isolation of MSCs from bone marrow, as it also contains blood cells at all stages of differentiation, as well as reticular cells. As the majority of blood cells are erythrocytes (red blood cells), which do not adhere in tissue culture, partial isolation of MSCs can be easily achieved as these cells do attach. As the other less adherent cells present will also be removed with medium changes, plating fresh marrow in tissue culture will in itself isolate the MSCs to a certain extent (Lazarus et al. 1997). These factors can be used as a simple method of separation of MSCs from marrow – after several passages any remaining haematopoietic cells, antigen positive for CD 45, 34 & 14, and macrophages disappear, leaving mesenchymal cells. Thus, as originally observed by Friedenstein, when whole bone marrow is cultured, characteristic spindle shaped MSCs adhere to the plastic forming colonies.

Haynesworth has investigated extensively the characteristics of MSCs *in vitro*, including the separation of MSCs from bone marrow, finding that a Percoll® gradient separates the marrow into three fractions based on density. On culturing the cells from each of these fractions, it was found that the low-density fraction, otherwise known as the 'buffy layer', contained MSCs (Haynesworth et al. 1992b).

Methods for separating the MSCs from bone marrow aspirates have been compared. Simple centrifugation of the marrow produced the buffy layer, but when this was compared with other methods it was found to produce the lowest yield of nucleated

cells (Connolly et al. 1989a). Ficoll®, a dense liquid not dissimilar to Percoll®, can be used to separate the cell types in marrow, using differential density centrifugation, whereby erythrocytes fall though the Ficoll® layer while mononuclear cells which are less dense remain on top. The yield of MSCs improved when the marrow was centrifuged at 400g for 30 minutes over a Ficoll® gradient, but was found to be greatest when the cells were allowed to separate by gravity during a 4 hour period over the gradient (Connolly et al. 1989a). However, in my study, due to practical implications of time and the risk of contamination, the MSCs were centrifuged over a Ficoll® gradient for 30 minutes, and it was determined that any other cells that remained in the culture would be removed due to their reduced ability to adhere to tissue culture plastic.

2.1.6 Yield of MSCs

By applying the stem cell colony theory first used by Friedenstein (that a colony of cells results from the division of a single cell), the number of MSCs present in a marrow sample can be determined by the number of colonies counted in the primary cultured marrow. The number of cell colonies has been found to be proportional to the number of nucleated cells originally isolated from the bone marrow (Majors et al. 1997), which suggests that a constant proportion of nucleated cells are MSCs. This has been quantified as 1 MSC per $1x10^5$ to $1x10^6$ mononucleated cells in marrow (Hicok et al. 1998). As tissue engineering requires a large number of cells to be delivered back into the patient, the small number of aspirated MSCs needs to be expanded and so proliferation of isolated MSCs will also been investigated in my study.

The number of MSCs in the marrow was found to correlate negatively with the age of the patient (Majors et al. 1997). However, no correlation was found between the growth rates of MSCs and the age of the donor when studied at a population level, with great variation in both these factors being observed between patients (Phinney et al. 1999). An explanation for this is that the density of MSCs within bone marrow decreases with age, but the growth rate of the individual MSCs does not. In view of this, throughout my thesis MSCs from each patient were used in both the control and test populations and the age of the patients from which aspirates were taken was limited to between 16 and 40 years.

2.1.7 Behaviour of MSCs in culture

The ability of bone marrow-isolated MSCs *in vitro* to form colonies does not persist after the first passage and it has been observed that the population cell growth follows lag, log and plateau phases (Goshima et al. 1991b). In the primary culture, both the lag and log phases last for longer periods of time, resulting in confluence after 12 days.

MSCs continue to proliferate in culture and maintain the potential to differentiate into osteoblastic cells for up to 38 population doublings (approximately passage 18) (Bruder et al. 1997b). Furthermore, there is evidence that, after passage 25, an increasing proportion of MSCs display apoptotic features, as noted by the loss of specific cell markers and integrins, which results in a reduction in the production of extracellular matrix proteins by these cells (Conget & Minguell 1999). As a result of these observations, in my studies, MSCs were only used up to passage 7.

2.1.8 Investigation of MSCs

Two experimental approaches have previously been used to research the potential of MSCs. In the first method, bone marrow has been investigated *in vivo* in animal models (Bab et al. 1988b). However, the experimental results of this research cannot be attributed to any specific cell type within the marrow and, furthermore, the effect of growth factors present within the animal cannot be quantified. Therefore, the second method of completing research *in vitro* has the advantage that the conditions can be easily controlled, although it is an artificial environment.

2.1.9 Markers of MSCs

Although it has proved difficult to identify a consistent marker for multipotent MSCs, as discussed in Chapter 1: Markers of MSCs section, Stro-1 has been suggested as a reliable marker (Gronthos et al. 1994). Therefore, Stro-1 was used in my study to aid confirmation of the isolation of MSCs from the bone marrow aspirates. The Stro-1 IgM mouse antibody labels MSCs, following which a fluorescent-labelled secondary antibody is attached to Stro-1 allowing the cells to be identified. Stro-1 has also been used to isolate MSCs by indirect fluorescence-activated cell sorting and these

separated cells have been shown to possess the potential to differentiate into osteoblastic cells (Stewart et al. 1999).

In this chapter, in addition to Stro-1 identification, the morphology and behaviour of the isolated cells will also be used as primary evidence of existence of MSCs.

2.1.10 Osteoblastic cell markers

One of the important characteristics of MSCs, which provides potential for the tissue engineering of bone, is the ability of these cells to differentiate into osteoblasts. This differentiation occurs in osteogenic culture conditions and can be measured by the presence of osteoblastic cell markers. The presence of osteoblasts in the differentiated culture indicates that the original isolated marrow cells contain committed osteoblast progenitor cells or MSCs that have been stimulated to differentiate via an osteoblast progenitor into osteoblasts, as there is evidence for such a pathway (Aubin et al. 1995). In this chapter, osteoblastic differentiation of bone marrow-isolated cells was investigated as evidence for their potential use in the tissue engineering of bone, as well as retrospective evidence for the isolation of osteoblast progenitor cells/MSCs.

Markers used to identify osteoblasts were discussed in Chapter 1: Osteoblastic cell markers section. In brief, the markers used in this chapter include: ALP, which is produced during early osteoblastic development and is widely used as a reliable marker of the osteoblast phenotype and cellular activity (Rodan et al. 1987), Osteopontin, which is an extra-cellular matrix protein that binds calcium to bone matrix, making it a sequentially later marker of osteoblastic maturation than ALP, and lastly Osteocalcin, which has been shown to be synthesised exclusively by mature odontoblasts and osteoblasts during mineralisation phase (Price 1985).

Initially, the specific messenger RNA (mRNA) for each of the markers was detected by a process of reverse transcriptase – polymerise chain reaction (RT-PCR). In brief, within cells mRNA is produced as a result of promoter signalling by a transcription factor, causing a gene sequence of DNA to be transcribed into mRNA, which is then translated into an amino acid sequence that is then folded into a protein. This results in the presence of mRNA before the protein can be detected. Detection of a specific

mRNA sequence within a cell involves extraction of RNA from cells, its conversion back to cDNA by the process of reverse transcriptase, followed by the addition of a specific gene primer and amplification of this sequence by polymerise chain reaction. Following this, the gene specific DNA can be separated and viewed on an electrophoresis gel identified by its size. This allows the protein specific mRNA sequences to be probed for and its presence within the cells was used as an indicator of cell phenotype. Biochemical assays were then also used in my study to detect the specific proteins produced by osteoblastic cells, thereby further characterising their phenotype.

As also detailed in Chapter 1, not only has Cbfa-1 been identified as a transcription factor that acts as a promoter for the transcription of osteocalcin (Ducy et al. 1997; Merriman et al. 1995), but there is also evidence that Cbfa-1 expression is essential for the mineralisation of bone (Komori et al. 1997). Therefore, it can be postulated that Cbfa-1 is expressed early in osteoblastic differentiation and maturation, before the osteocalcin gene is expressed. The presence of Cbfa-1 gene expression may also be essential for osteoblastic differentiation of cells and so the expression of Cbfa-1 was also investigated in this chapter.

2.1.11 In this chapter

In summary, for MSCs to be used for the tissue engineering of bone, which is the overall aim of my thesis, firstly it is necessary to show that MSCs can be isolated from human bone marrow aspirates. Therefore, aspirates were taken from the iliac crest of patients, an accessible source of red marrow, and a Ficoll® gradient was used to separate the MSCs. The behaviour of the cells was observed in culture and Stro-1 was used as a marker to identify the presence of MSCs. As their name suggested, MSCs are multipotent cells with the ability to differentiate down the mesenchymal cell lines. Hence, as further evidence of their multipotent potential marrow-derived cells were stimulated to differentiate into osteoblasts, chondrocytes and adipocytes.

For MSCs to be useful in the tissue engineering of bone, it was specifically necessary to demonstrate their ability to differentiate into osteoblasts. This was investigated more

closely by culturing half the MSCs from each aspirate in osteogenic supplements, using markers of osteoblasts to compare the two populations.

As discussed above, due to the low density of MSCs in marrow, the use of these cells for clinical treatments requires their proliferation in culture and so expansion of these cells in culture was also investigated. A further aim of this chapter was to detail the methods used throughout this thesis, for the isolation and culture of MSCs from bone marrow and the identification of osteoblastic cells.

2.1.12 Hypothesis

Therefore, the hypotheses of this chapter were:

1. MSCs can be isolated from human bone marrow aspirates;
2. When cultured in monolayer and in the presence of appropriate supplements, the marrow-isolated cells differentiate into osteoblasts, adipocytes and chondrocytes, confirming the presence of MSCs.

2.2 MATERIALS and METHODS

2.2.1 Materials

General

Bone marrow aspirate needles (Rocket Medical 131000)
Ficoll (Amersham Pharmsin Biotech 75285)

Cell culture

Dulbecco's modified phenol red free medium (Sigma D5921)
Trypsin Hepes 0.25% (Sigma T8003)
Dulbecco's phosphate buffered saline (PBS) (Sigma D8662)
Trypan blue solution (Sigma T8154)
Haematocytometer (Sigma Z35952-9)

Standard medium

Dulbecco's modified eagles medium (Sigma D6429) 500ml
Foetal calf serum (Sigma F7524) 500ml
Penicillin 50U/ml (Sigma P0906)
Streptomycin 50μg/ml (Sigma P0906)

Additional supplements added to standard medium:

Osteogenic supplements (OS)
Dexamethasone 10^{-7}M (Sigma D1756)
Ascorbic acid 50μM (Sigma A4544)
β-glycerol phosphate 10mM (Sigma G6376)

Adipocytic supplements
Dexamethasone 10^{-6}M (Sigma D1756)
Insulin 10g/ml (Sigma I0516)
Indomethacin 100mM (Sigma I7378)

Chondrocytic supplements
Transforming growth factor-β (TGF-β) 10ng/ml (Sigma T7039)
Dexamethasone 10^{-7}M (Sigma D1756)
Ascorbic acid 50ng (Sigma A4544)
Sodium pyruvate 1nM (Sigma P5280)

Histological stains

Formaldehyde (BDH Chemicals)
Haematoxylin (BDH Chemicals 340374T)

ALP: Napthol AS-B1 sodium phosphate
New Fuchsin 4% in HCL (Sigma)
Sodium nitrate (Sigma S2252)
Napthol AS-B1 phosphate (Sigma N2250)
Methyl-green counter stain (HD Supplies 465)

Calcification: Von Kossa
Silver nitrate 2% (Sigma S2252)
Sodium thio-sulphate 2.5% (BDH Chemicals 10268)
Neutral red 1% (Sigma N6634)

Lipid: Oil Red O
Oil Red O (RA Lamb S2672)
Absolute isopropyl alcohol (BDH Chemicals 296946H)
Dextrin (Sigma D2256)
Mayer's Haemalum Solution (dissolved in 1 litre distilled water):
Haematoxylin (BDH Chemicals 340374) 10ml
Sodium Iodate (Sigma S4007) 0.2g
Alumininium potassium sulphate (BDH 27085 SR) 50g
Chloral hydrate (BDH 276684R) 1g
Citric acid (BDH Chemicals 100813M) 1g

Collagen: Alcian Blue/Sirius Red
Alcian blue (HD Supplies)
Molybdophosphate acid (BDH Chemicals)
Sirius red (HD Supplies)

Stro-1 antibody

Stro-1 supernatant mouse IgM (Developmental Studies Hybridoma Bank, University of Iowa, US)
FICA anti-mouse IgM (Sigma F9259)

SEM (Scanning Electronic Microscopy)

Sodium cacodylate (BDH Chemicals 30118)
Glutaraldehyde (Agar scientific R1011)
Osmium tetroxide 2% (Agar Scientific R1022)
Ethyl alcohol (James Burrough)
Hemamethyldisaiazane (Agar Scientific)
Aluminium stubs 32mm diameter (Agar Scienitific 9318)
Tannic acid (Sigma T0125)

RNA

RNAeasy mini kit (Qiagen 74104)
dNTP's (Promega U1240)
Oligo dT Primers (Promega C1101)
RNAsin (Promega M2111)
RNA polymerase (P1085)
MMLV-RT (Gibco Life technologies 28025-013)
Amphi Taq (Applied Biosystems N808 0158)
Primers (Severn Biotech)
Ethidium bromide (Sigma E1510)
TAE buffer (Sigma T9650)

Assays

Alkaline phosphatase assay (Randox EC 3131)
Human Osteopontin enzyme immunometric assay (Metachem Diagnostics 90027)
Osteocalcin OSCA test (B.R.A.H.M.S. 57.1)
DNA assay Hoeschst 33258 (Sigma B2883)

2.2.2 MSC Isolation methods

2.2.2.1 Bone marrow aspiration

Local ethical committee approval was gained for the procedure of bone marrow aspirations from patients undergoing orthopaedic operations under general anaesthetic at the Royal National Orthopaedic Hospital. Following informed consent, bone marrow aspirations were obtained from the posterior iliac crest of patients. The age of the patients ranged from 16-40 years.

The procedure was performed while the patients were under general anaesthetic for elective orthopaedic procedures, just before the start of the operation. The patients were placed in the left lateral position and the skin over posterior iliac crest was cleaned with chlorhexidine and draped. Under sterile conditions, using a bone marrow aspiration needle, 2ml of bone marrow was aspirated from the intramedullary cavity of the ilium. This volume of marrow has been shown to harvest 85% of the stem cells from the area aspirated (Muschler et al. 1997). As the volume of the bone marrow aspirated increases, the percentage of stem cells decrease as venous blood starts to be aspirated in addition to marrow. To maximise the harvest of marrow, the plunger on the syringe is pulled back sharply, multiple times, as this technique draws the marrow out of the surrounding trabeculae.

The aspirate was taken from the posterior iliac crest 5cm lateral to the sacro-iliac joint. The marrow was aspirated into a syringe, which contained 1ml of 5000 units of heparin to prevent clot formation. The mixture of marrow and heparin was then placed into a sterile universal container and mixed.

2.2.2.2 Processing of bone marrow sample

The MSCs were separated from the other cells present in bone marrow by density centrifugation using a Ficoll® gradient. The aspirate was slowly added to 3ml of Ficoll where the aspirate formed a separate layer on top of the Ficoll®, which was centrifuged at 400g (1510 rpm) for 30 minutes. The sample separated into 4 distinct layers with haematopoietic cells at the bottom, a clear Ficoll® above this and serum upper most. A fourth layer, known as the buffy layer, containing mononuclear cells including MSCs, formed at the interface between the Ficoll® and serum, and this layer was carefully harvested using a pipette.

The mononuclear cells were washed with standard medium and centrifuged at 1000g (2000 rpm) for 10 minutes, resulting in the formation of a cell pellet, which was collected and resuspended in medium and centrifuged again at 1000g for 5 minutes, resulting in a cell pellet that contained the MSCs. This pellet was resuspended in 2ml of medium through a 23-gauge needle and plated in two 75 cm^2 flasks, which were placed in an incubator at 37°C and 5% CO^2 to culture the cells. These incubator conditions were used throughout the studies in this thesis. A total of twelve patients' bone marrow aspirations were used in the experiments within this chapter.

2.2.2.3 Cell culture

The MSCs adhered to the tissue culture plastic and the culture medium was changed every 3 days to remove any remaining non-adherent, haematopoietic cells. The cells were cultured until confluence, at which point the cells were trypsinised and divided into 3 culture flasks (passage 1).

To trypsinise the cells, the medium was removed and the cells were washed with 5ml of phosphate buffered saline (PBS). A volume of 8ml of 10% trypsin (with 1% hepes

buffer) was added to the cells and agitated for 5 minutes. Any remaining adherent cells were removed following brief use of a cell scraper. The trypsin containing cells in suspension was removed and the flask was washed twice with standard medium. These fluids were placed in a universal container and centrifuged at 2000 rpm for 5 minutes. As a result of this, a cell pellet formed which was resuspended and re-plated into 3 new culture flasks. This method of trypsinisation was used to divide confluent flasks of cells throughout my thesis.

In order to count the number of cells used in each experiment throughout my thesis, trypan blue was used to stain the cells, distinguishing dead cells as these membranes allowed reagent through, staining the whole cell blue. A haemocytometer was used to count cells ensuring that the same number of MSCs was used in each sample.

2.2.3 Characterisation of MSCs

2.2.3.1 Observation of cells in culture

The morphology and behaviour of all the isolated bone marrow cells in monolayer was regularly observed under light microscopy.

2.2.3.2 Stro-1 antibody marker

The monoclonal IgM murine antibody, Stro-1 was used to identify adult MSCs. For each of four patients' marrow-isolated cells, eight glass autoclaved cover slips were cultured with 25,000 cells on each for 48 hours. The first two cover slips were cultured with isolated marrow cells in standard medium and, on the second two cover slips, cells were cultured in with osteogenic supplements (OS), which was made up of standard medium with the addition of dexamethasone (10^{-7}M), ascorbic acid (50μM) and β-glycerol phosphate (10mM) - the same concentrations were used throughout my thesis for OS. Two different osteosarcoma cell lines were cultured as controls on the remaining four cover slips. One of the osteosarcoma cell lines, MG 63 cells, was a positive control, as this cell line is known to express Stro-1 and Saos-2 cells were used as a negative control, as these are known not to express Stro-1 (Stewart et al. 1999).

After 48 hours in culture the medium was removed and the cells were washed four times with PBS. Following this 1ml of 1:6 diluted Stro-1 monoclonal antibody was added to the cells and the cells were then incubated at 37°C for 1.5 hours. The primary antibody was removed and replaced with the goat anti-mouse IgM fluorescent-labelled antibody (FICA) and the cells were incubated for a further hour. Following six further washings with PBS, the presence of cell labelling was observed under the fluorescent microscope, the relative intensity of fluorescence between cell types was compared and photographed.

2.2.4 Mesenchymal lineage differentiation – Histological stains

2.2.4.1 Osteoblastic differentiation

2.2.4.1.1 ALP stain: Naphtol-AS-B1 sodium phosphate

Two autoclaved glass cover slips were seeded with 20,000 marrow-isolated cells and cultured for 7 days in standard medium. The same numbers of cells were also cultured on two cover slips with the addition of OS, for 7 and 14 days. This was repeated with cells isolated from the marrow of five patients.

At the end of the culture period, the medium was removed and the cells were fixed with 2% formaldehyde and stained for ALP with Naphtol-AS-B1 phosphate. Equal volumes of 4% New Fuchsin in 2M HCl and 4% sodium nitrate, followed by buffer (0.2M Tris with 0.1M HCl) and Napthol AS B1 phosphoric acid were added to stain the cells. These were then counter stained with fast red violet and the mounted cells were observed and photographed under the light microscope. After the same culture period, 2 repeats of the Saos-2 osteosarcoma cells, cultured in standard medium as a positive control, were stained for ALP expression using the same method.

2.2.4.1.2 Calcification stain: Von Kossa

Von Kossa stain was used to identify extra-cellular calcium produced by osteoblasts during the mineralisation phase.

Four glass cover slips were each seeded with 250,000 cells and two were cultured for 21 days and two for 28 days, for each time period, one in the presence and one in the absence of OS. This was repeated for bone marrow-derived cells of four patients. Saos-

2 osteosarcoma cells were again used as a positive control and two repeats were used for each condition. After the culture period, the medium was removed and the cells were placed in 2% silver nitrate and exposed to bright light until the control blackened. After two washes with distilled water, the cells were treated with 2.5% sodium thio-sulphate and then counter stained with 1% neutral red. Following mounting on slides, the cells and mineralisation stained black were observed under light microscopy and photographed.

2.2.4.2 Adipocytic differentiation

2.2.4.2.1 Lipid stain: Oil Red O

Marrow-isolated cells were grown on 8 autoclaved glass cover slips at a seeding density of 50,000 cells per cover slip. Cells on half of the cover slips were cultured in standard medium and the remaining half were cultured in adipocytic medium. Adipocytic medium consisted of standard medium with the addition of insulin (10g/ml), dexamethasone (10^{-6}M) and indomethacin (100mM). Two cover slips from each condition were then cultured for 7 days and the other two for 14 days. This was repeated for each of 4 patients' marrow samples.

Following these culture periods, the cells were stained for intracellular lipid using oil red O stain. Firstly, the medium was removed and the cells were rinsed with PBS and fixed in 10% formaldehyde for 5 minutes. This was rinsed off with distilled water and the cover slips were placed in glass boats filled with oil red O stain for 20 minutes. The cover slips were washed briefly under running water to remove excess stain and counterstained with Mayer's haemalum for 3 minutes, following which any excess stain was rinsed off. The cover slips were air-dried, mounted on slides and cells were visualized under light microscopy. As a result of this staining process, lipid droplets within the cells were stained red and the nuclei blue.

2.2.4.3 Chondrocytic differentiation

2.2.4.3.1 Collagen: Alcian Blue/Sirius Red

Similarly to the methods described above, 50,000 marrow-isolated cells were cultured on six cover slips for 14 days; pairs of cover slips were cultured in standard, osteogenic and chondrocytic medium, and a further two cover slips were cultured with

bovine chondrocytes in standard medium as a positive control. This was repeated for the isolated marrow cells from four patients. The chondrocytic supplements that were added to standard medium were TGF-β (10ng/ml), dexamethasone (10^{-7}M), ascorbic acid (50ng) and sodium pyruvate (1nM).

After the culture period, the cells were stained on cover slips for collagen using alcian blue/sirius red. The cells were rinsed in PBS and placed in a glass boat, into which the stain solutions were added, with distilled water rinses in between each solution. The first solution used was Weigert's haematoxylin and the second was alcian blue. The cells were stained for 10 minutes in each of these. Following this, the boat was filled with molybdophosphatic acid for 20 minutes. This was removed and the cells were stained with sirius red for 60 minutes, after which the cells were washed for a final time in distilled water, air dried and mounted on slides. In order to visualize the collagen lattice, the slides were observed under polarized light and photographs were recorded.

2.2.5 Further investigation of osteoblastic differentiation

The effect of OS on MSCs was investigated by culturing marrow-derived cells in standard medium, to act as a control, compared with culture in osteogenic medium. The following factors were used to characterise the osteoblastic phenotype:

1. Morphology observed under light microscopy
2. Morphology under SEM
3. Histological staining (as above)
4. Messenger RNA expression
5. Protein production

2.2.5.1 Osteoblastic differentiation - Morphology

2.2.5.1.1 Light microscopy

Bone marrow-derived cells in culture were observed regularly under light microscopy, to assess their growth and morphology. Photographs were taken of both control cells cultured in standard medium and of cells cultured with OS.

2.2.5.1.2 Scanning electron microscopy (SEM)

Therminox discs were seeded with 25,000 cells and cultured in 24-well plates. Three discs were cultured in OS and three in control medium, to compare the effect of osteogenic factors on marrow-isolated cells. After 1 and 7 days in culture the discs were processed for scanning electron microscopy (SEM). This was repeated for cells derived from the bone marrow of five patients.

The processing for SEM firstly involved removing the therminox discs from the incubator and cooling them at 4°C, before replacing the medium with 1.5% glutaraldehyde in 0.2M sodium cacodylate buffer. The processing then consisted of a series of incubations in various solutions to stain the cells and dehydrate the sample. Firstly, the glutaraldehyde was removed and any remaining was washed off by covering the samples with 0.2M sodium cacodylate buffer for 60 minutes. Following this, the samples were placed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 60 minutes, to stain the cellular material. This is a strong oxidising agent so particular care was used with this substance, handling only while wearing gloves in the fume hood.

The osmium was removed and the samples were washed three times with 0.1M sodium cacodylate buffer. Each wash was left for 5 minutes to allow equilibration. The samples were then placed in 1% tannic acid in 0.05M buffer for a further 60 minutes, after which this solution was similarly removed with three buffer washes. The samples were dehydrated by placing them in increasing concentrations of alcohol. From 20% ethyl alcohol, the concentration of alcohol was serially increased in 10% increments to 100% ethyl alcohol, allowing equilibration with each concentration of alcohol. The samples were dried in hexamethyldisiazane, air dried, mounted on aluminium stubs and coated with gold/palladium in argon for 2 minutes.

The cells on the therminox discs were observed under the scanning electron microscope (Joel Winsem JSM-35C 6300) and the morphology of the cells was compared between those that had been cultured in osteogenic and control conditions, after days 1 and 7 in culture.

2.2.5.2. Messenger RNA (mRNA) analysis of cells

The expression of various genes normally expressed by pre-osteoblasts and osteoblasts was compared between marrow-isolated cells from three patients cultured in standard medium and OS after 0, 1, 3, 5 and 7 days. Saos-2 osteosarcoma cells were again used as a positive control.

The expression of 2 genes osteopontin and osteocalcin, which are expressed by osteoblastic cells during the stages of maturation, was investigated. Cbfa-1 expression was studied as a marker of osteoblast progenitor cells and GAPDH (a house keeping gene) was used as a positive control for each experiment.

The primer sequences used to assess the gene expression by these cells were:

Cbfa-1	5' TCT TCA CAA ATC CTC CCC 3'	230 bp
	5' TG ATT AAA AGG ACT TGG TG 3'	

(Christiansen et al. 2000)

Osteopontin	5' CCA AGT AAG TCC AAC GAA AG 3'	347 bp
	3' GGT GAT GTC CTC GTC TGT A 5'	

(Rickard et al. 1996)

Osteocalcin	5' CAT GAG AGC CCT CAC A 3'	310 bp
	3' AGA GCG ACA CCC TAG AC 5'	

(Rickard et al. 1996)

GAPDH	5' ACC ACA GTC CAT GCC ATC AC 3'	452 bp
	5' TG ATT AAA AGG ACT TGG TG 3'	

(Christiansen et al. 2000)

ALP	5' ACG TGG CTA AGA ATG TCA TC 3'	475 bp
	3' CTG GTAG GCG ATG TCC TTA 5'	

(Phinney et al. 1999)

2.2.5.2.1 RNA extraction

Confluent flasks of marrow-derived cells were trypsinised and the number of cells in each was counted using a haematocytometer. One million cells were resuspended and centrifuged at 2000 rpm to form a cell pellet, from which RNA was extracted using a Qiagen RNA easy kit. The amount of RNA extracted was then measured and the yield of mRNA was found to vary between 200 - 400μg/ml in 50μml volume.

2.2.5.2.2 Reverse Transcriptase reaction

Reverse Transcriptase reaction synthesized cDNA from 2μg of mRNA in a 50μl volume, with dNTP's 200μM, Oligo dT 0.25μg, MMLV-RT 200U/μg RNA, RNAse inhibitor 50U/ml and buffer 10mM. The reaction time cycle was 10min at 25°C, 60 min at 42°C and 5 min at 94°C.

2.2.5.2.3 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was carried out on 10μl of the sample obtained as a result of the reverse transcriptase reaction, in a total volume of 50μl. The PCR reaction contained dNTP at 200mM, specific primer at 0.5μl (50pmol) for each sense and anti-sense, ampli taq DNA polymerase at 2.5U/100μl, 5μl buffer and 3μl $MgCl_2$. After an initial denaturation at 94°C for 2 minutes, amplifications were performed for 30 cycles of 94°C for 30 seconds, 55°C for 2 minutes, 72°C for 2 minutes, for ALP, osteopontin and osteocalcin primers, 36 cycles of 94°C for 2 minutes, 55°C for 2 minutes, 72°C for 2 minutes for Cbfa-1 and GAPDH primers, and 35 cycles of 94°C for 30 seconds, 59.4°C for 45 seconds, 72°C for 2 minutes, for BSP primer, which were followed by 10 minutes at 72°C.

2.2.5.2.4 Electrophoresis gel

An electrophoresis gel containing 2% agarose and 5μl ethidium bromide was set up in specific wells, into the first of which a DNA ladder was placed, so that the DNA bands could be compared against a ladder of known base pairs. Subsequent wells were used for each primer sequence for MSCs and MSCs in OS for each day. After the current had been past though the gel for 30 minutes the bands were photographed under UV light.

2.2.5.3Biochemical protein assays

2.2.5.3.1 Culture scheme for protein assays

1. ALP/DNA: 25,000 cells in 24-well plates, 2 repeats for each of 7 patient samples for both control and osteogenic conditions, for days 5, 10 and 15 in culture.

2. ALP/DNA: 250,000 cells in 12-well plates, 2 repeats for each of 5 patient samples for both control and osteogenic conditions, after 14 and 28 days in culture.
3. Osteopontin/DNA: 250,000 cells in 12-well plates, 2 repeats for each of 6 patient samples for both control and osteogenic conditions, for 14 days in culture.
4. Osteocalcin/DNA: 25,000 cells in 24-well plates, 2 repeats for each of 6 patient samples for both control and OS, for 15 days in culture.
5. Osteocalcin/DNA: 250,000 cells in 12-well plates, 2 repeats for each of 6 patient samples for both control and OS, for 28 days in culture.

2.2.5.3.2 Alkaline phosphatase assay (ALP)

The amount of ALP protein produced by the cells within each sample was measured using a biochemical assay adapted from Oreffo (Oreffo et al. 1998). As the isoenzyme is intracellular and membrane bound (Ecarot-Charrier et al. 1983), the assay was performed on the cell lysate. The cells were lysed by replacing the medium with 1ml of distilled water, then freezing them to –70°C and warming them to 37°C twice. The assay measures the amount of ALP by assessing the amount of p-nitrophenol cleaved from p-nitrophenol phosphate at alkaline pH (1M diethanolamine buffer). The resulting colour change can then be measured at 405nm absorbance using the COBAS-BIO (Roche, UK) centrifugal analyser.

2.2.5.3.3 Osteopontin assay

Osteopontin produced by the cells was detected and quantified using an enzyme immunometric assay. As osteopontin is an extra-cellular protein, this assay was performed on the medium removed from the cells while in culture. The assay uses a monoclonal antibody to bind to the osteopontin, labelled with Horseradish peroxidase. A substrate buffer was then added to bind to the monoclonal antibody generating a colour. The resulting degree of colour change, measured at 450nm, correlated with the amount of osteopontin protein when compared with the generated standard curve.

2.2.5.3.4 Osteocalcin assay

The Osteocalcin assay method was adapted from Power (Power & Fottrell 1991), whereby the quantity of osteocalcin was determined by a competitive

radioimmunoassay, using antibody-coated tubes and I^{125} labelled osteocalcin. During incubation the osteocalcin in the samples competed with the radioactively labelled osteocalcin in the assay for binding sites on the coated tubes. A high osteocalcin concentration in the sample led to a low binding of the labelled antibody, resulting in an inverse relationship between the amounts of radioactively labelled and sample osteocalcin bound to the coated tubes. After the unbound antibody was removed, the radioactivity was measured using a gamma counter. The readings were compared with a standard curve that was generated from known concentrations of osteocalcin and the concentrations of osteocalcin in the samples were calculated.

2.2.5.3.5 DNA assay

Although the same numbers of cells were seeded in each well for the protein assays at the beginning of the experiments, the growth rate of the cells may have varied between well-samples over the culture period, resulting in a difference in cell number. As protein production is relative to the number of cells present in culture, standardisation for possible variations was necessary to allow comparisons to be drawn between different conditions. This was achieved by measuring the total DNA content of each sample, as this is proportional to cell number. Thus, the amount of protein produced (ALP, osteopontin and osteocalcin) by each sample of cells was divided by the total DNA content of that sample, thereby allowing statistical comparisons to be made between the different groups. This method of standardising protein measurements to allow comparison between samples was used throughout my thesis.

A DNA assay, based on methods described in the literature (Rago et al. 1990; Rao & Otto 1992) was used, in which DNA content was measured on cell lysates using a fluorimetric dye, Hoechst 33258. The dye is specific for DNA as it binds to contiguous adenine-thymine base pairs resulting in the emission of fluorescence at a wavelength of 460nm. After the addition and mixing of Hoechst reagent to the cell lysates, the degree of fluorescence was measured using an Ascent plate reader (Labsystems). DNA values were calculated as a result of comparisons with known DNA values used to create a standard curve.

2.2.5.3.5 Statistical analysis

Production of biochemical proteins was measured to assess the effect of culturing marrow-derived cells in OS medium compared with those in standard medium. The results were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk statistical tests, but the results were not found to follow a normal distribution. As a result non-parametric, Mann Whitney U tests were performed to compare the results of the two culture conditions.

2.2.6 Measurement of Proliferation Rate

2.2.6.1 DNA assay

The change in total level of DNA content, measured using the Hoechst 33258 assay as described above, was used to assess the proliferation rate of marrow-derived cells over time. Firstly, 25,000 cells from seven patients' marrow aspirates were seeded and cultured in either standard or OS, with two repeats for each condition, for 15 days. At 5-day time intervals a total of 28 samples were analysed for total DNA content using Hoechst DNA assay, as described above. The proliferation rate of the cells in culture was assessed by comparing the DNA content between consecutive days, in each condition. Furthermore the effect of OS on the proliferation rate was also assessed by comparing the DNA content of samples at the same time point, but cultured in different conditions. Non-parametric statistical tests were used to compare the groups, as the results were not found to follow a normal distribution.

A similar experiment was conducted to support the first results, where 250,000 marrow cells from 6 patients were cultured over 28 days, in both standard and osteogenic medium, using 2 repeats per sample, per condition. After 14 and 28 days the cells were lysed and the total DNA content was measured using Hoechst assay.

2.3 RESULTS

2.3.1 Characteristics of bone marrow derived cells

2.3.1.1 Cell culture – light microscopy observations

Initially, the isolated bone marrow cells were noted to form a cloudy suspension in the culture flasks, however this cleared following two medium changes. From every bone marrow aspirate taken, after an average of 7 days in culture, colonies of cells containing up to of 500 similar cells were observed to form (see figure 1.1a).

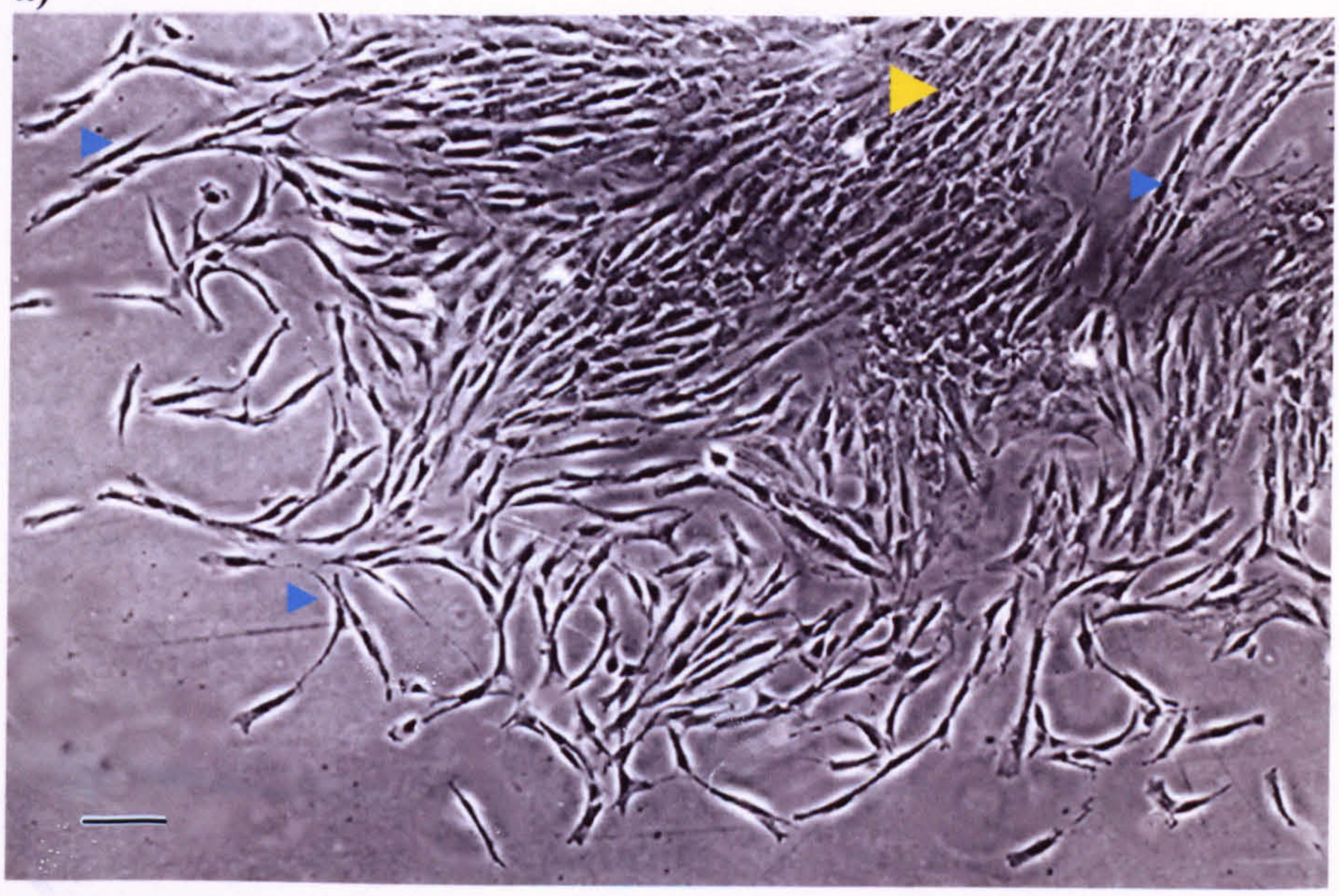
This initial tendency for the cells to form colonies was only observed in the primary marrow cultures. The cells grew in number resulting in confluent cultures after 12 to 14 days (see figure 1.1b).

Following each passage, the cells were re-plated at a constant density and after passage 1 and 2 the cells were observed to become confluent after an average of 10-12 days. Subsequent to passage 3, the cells were noted to become confluent after 6-8 days in culture.

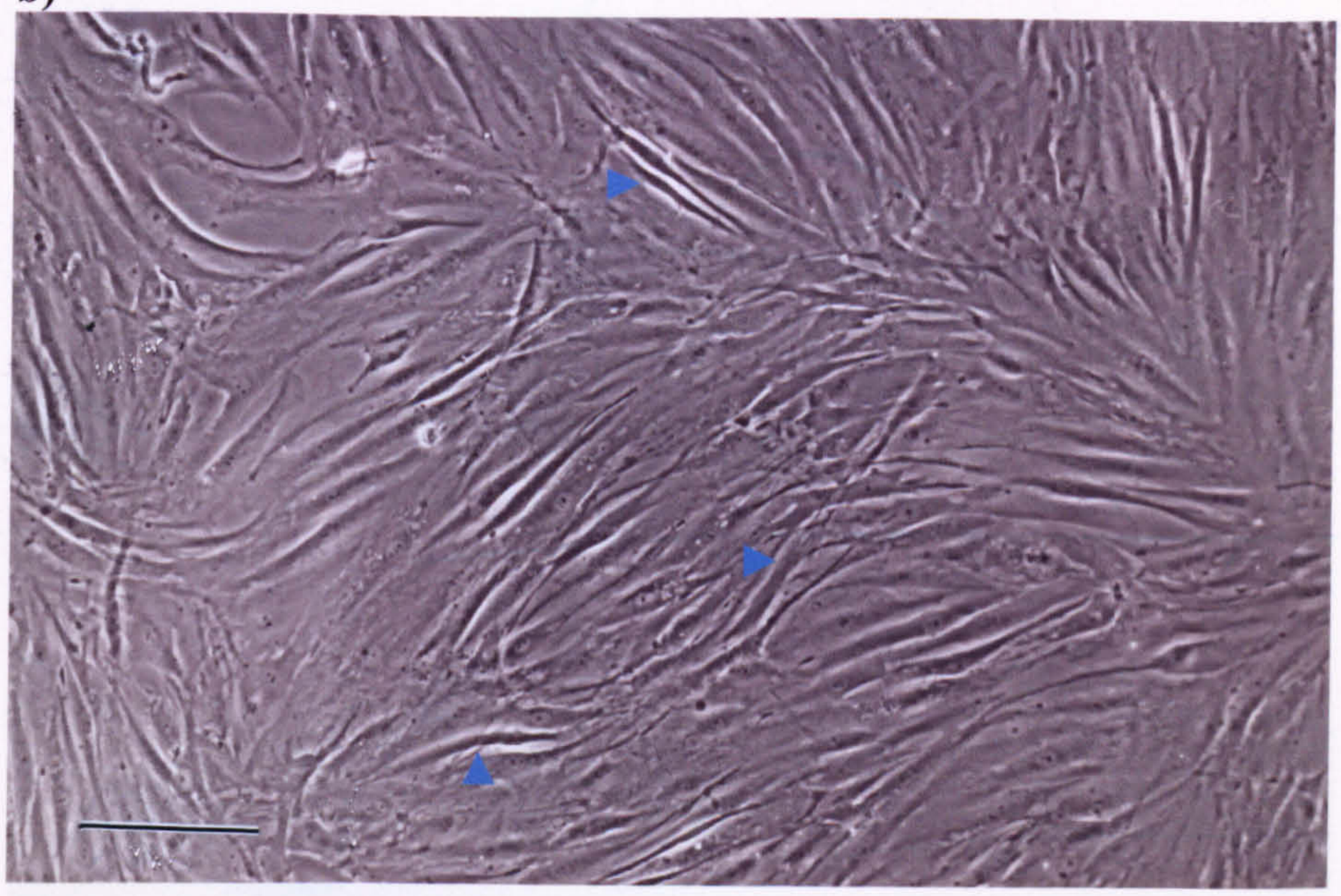
The adherent cells were observed to be long and spindle-like in shape, similar to fibroblasts and this morphology was observed to persist for 10 passages.

Figure 1. 1: Light microscopy picture of marrow-derived cells, a) after 7 days in culture, showing part of a cell colony with the centre of the colony indicated by the yellow arrow, bar = 200µm, b) after 14 days, showing a confluent culture. Blue arrows show spindle-like cells, bar = 200µm.

a)



b)



2.3.1.2 Antibody markers for MSCs

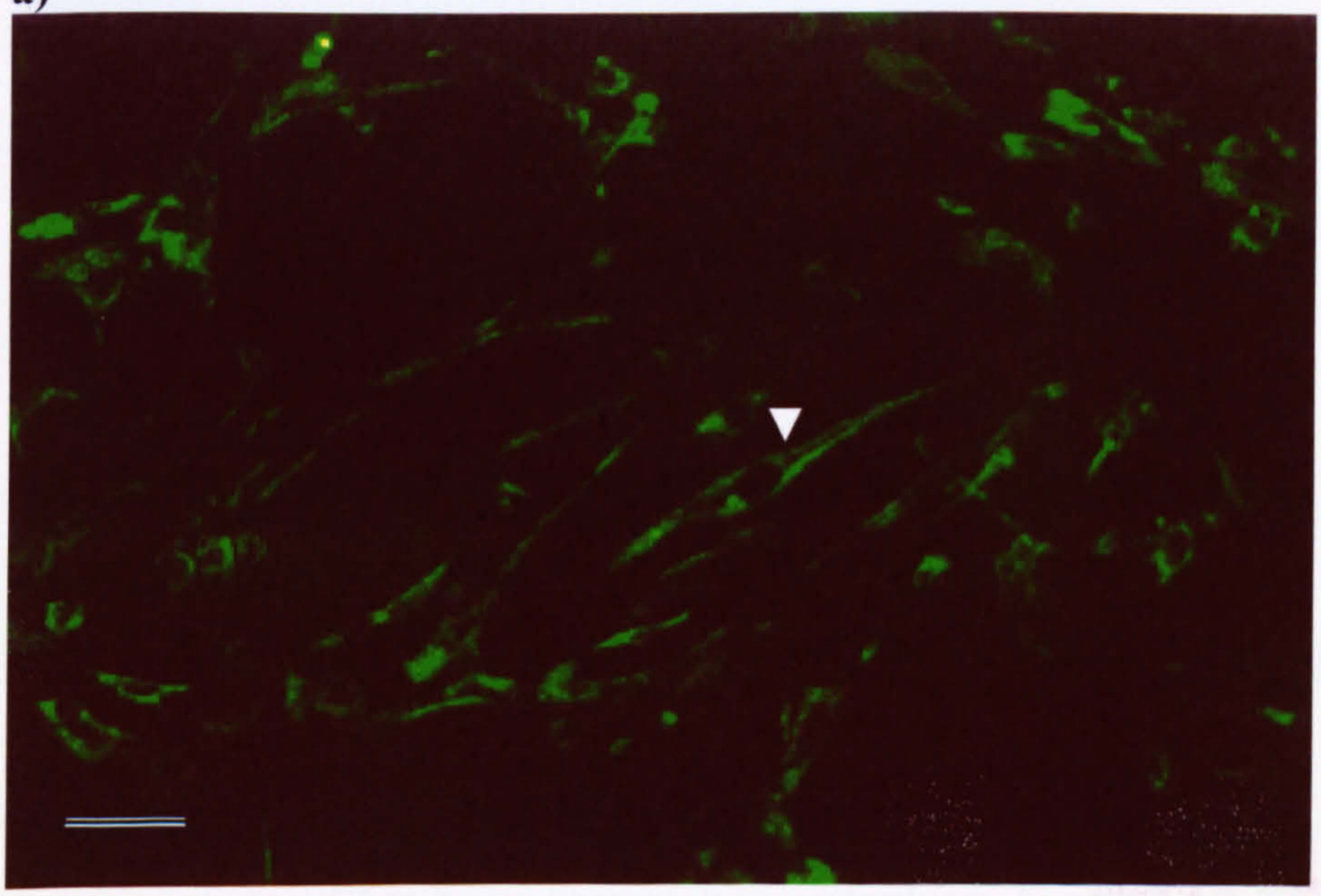
The marrow-derived cells were tested with Stro-1 monoclonal antibody, a marker for MSCs. Cells from every patient cultured in standard medium labelled positively for the antibody and the spindle shape of the cells could be determined through the immuno-fluorescence, as the antibody labels a membrane-bound antigen (see figure 1.2a).

The cells cultured in OS for 48 hours prior to antibody labelling were noted to change in shape, becoming squarer with increased labelling of the cytoplasm (see figure 1.2b).

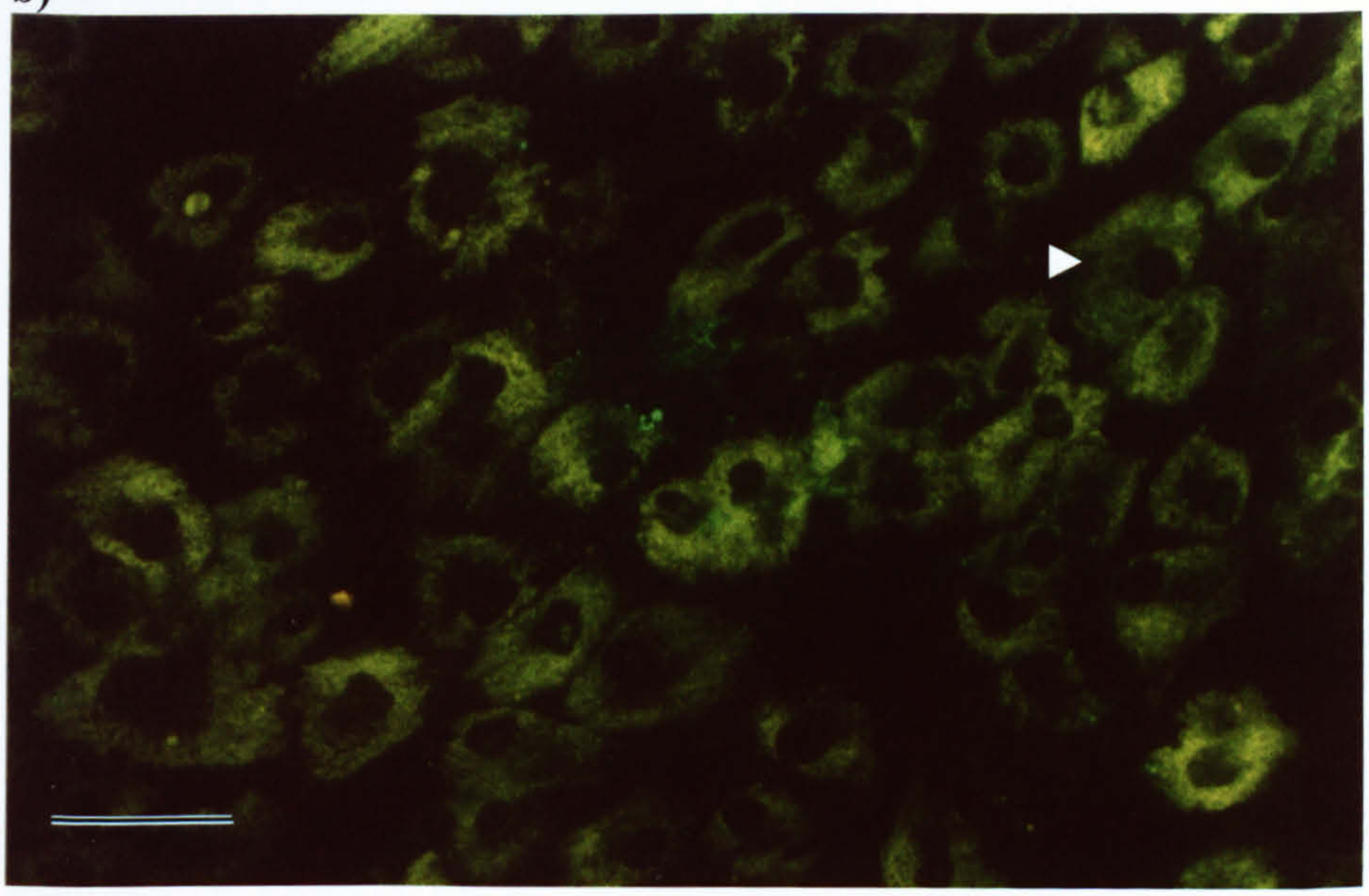
The positive control, MG63 osteosarcoma cells, were labelled by the Stro-1 antibody, but the intensity of the fluorescence was much less than for the marrow cells and the negative control, Saos-2 osteosarcoma cells, were not fluorescently-labelled by Stro-1 and therefore no photographs were taken.

Figure 1. 2: Stro-1 immuno-fluorescence marker identified marrow-derived cells from every sample tested after 48 hour, a) standard medium, spindle-shaped cells fluorescently-labelled shown by arrow, b) osteogenic medium, labelled squarer cell shown by arrow and c) weakly labelled MG 63 osteosarcoma cells in standard medium, shown by arrow, each bar = 100μm.

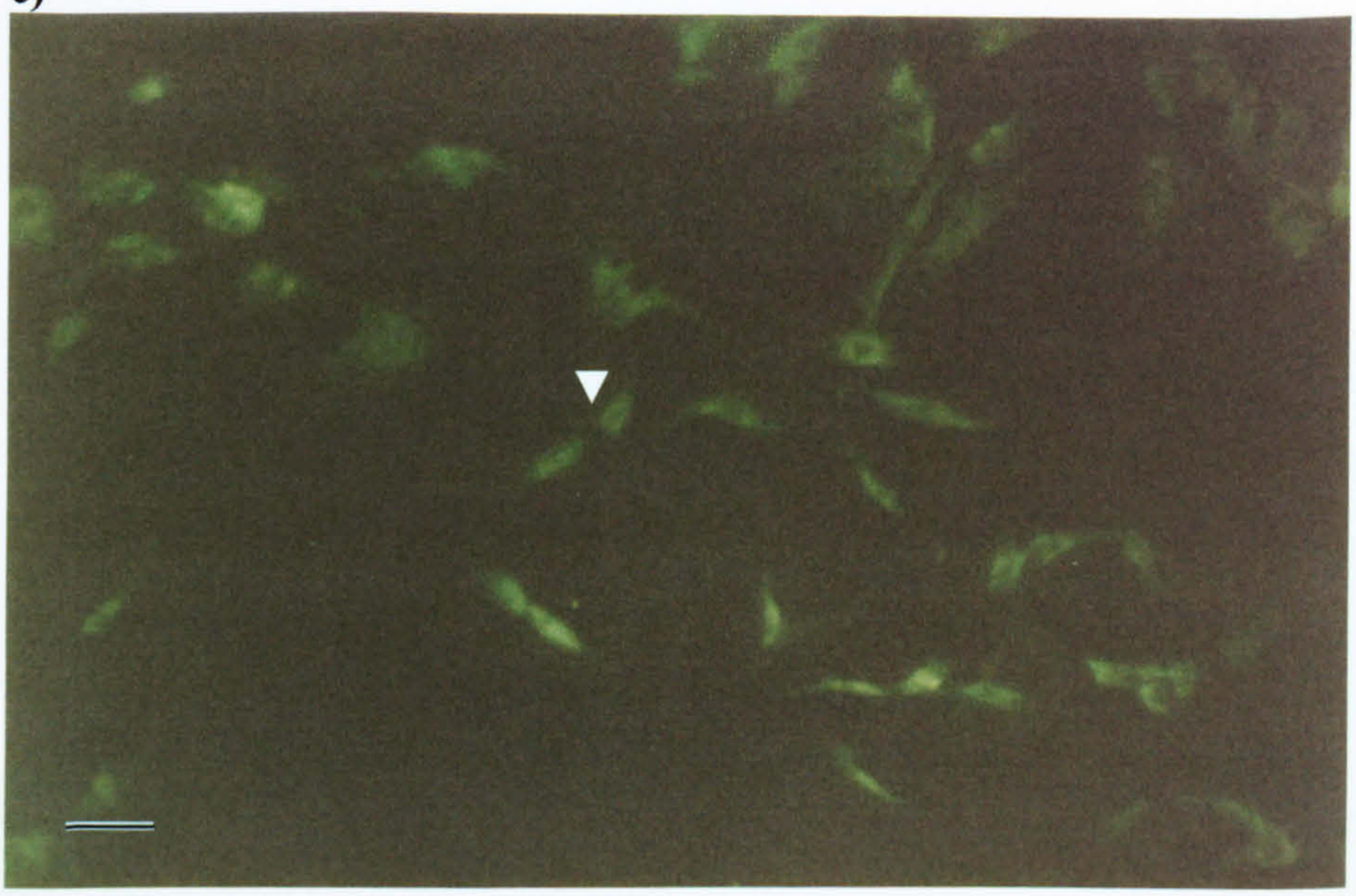
a)



b)



c)



2.3.2 Observations of marrow cells stimulated to differentiate down mesenchymal cell lines

2.3.2.1 Osteoblastic differentiation – Napthol – AS-B1 sodium phosphate Histology stain

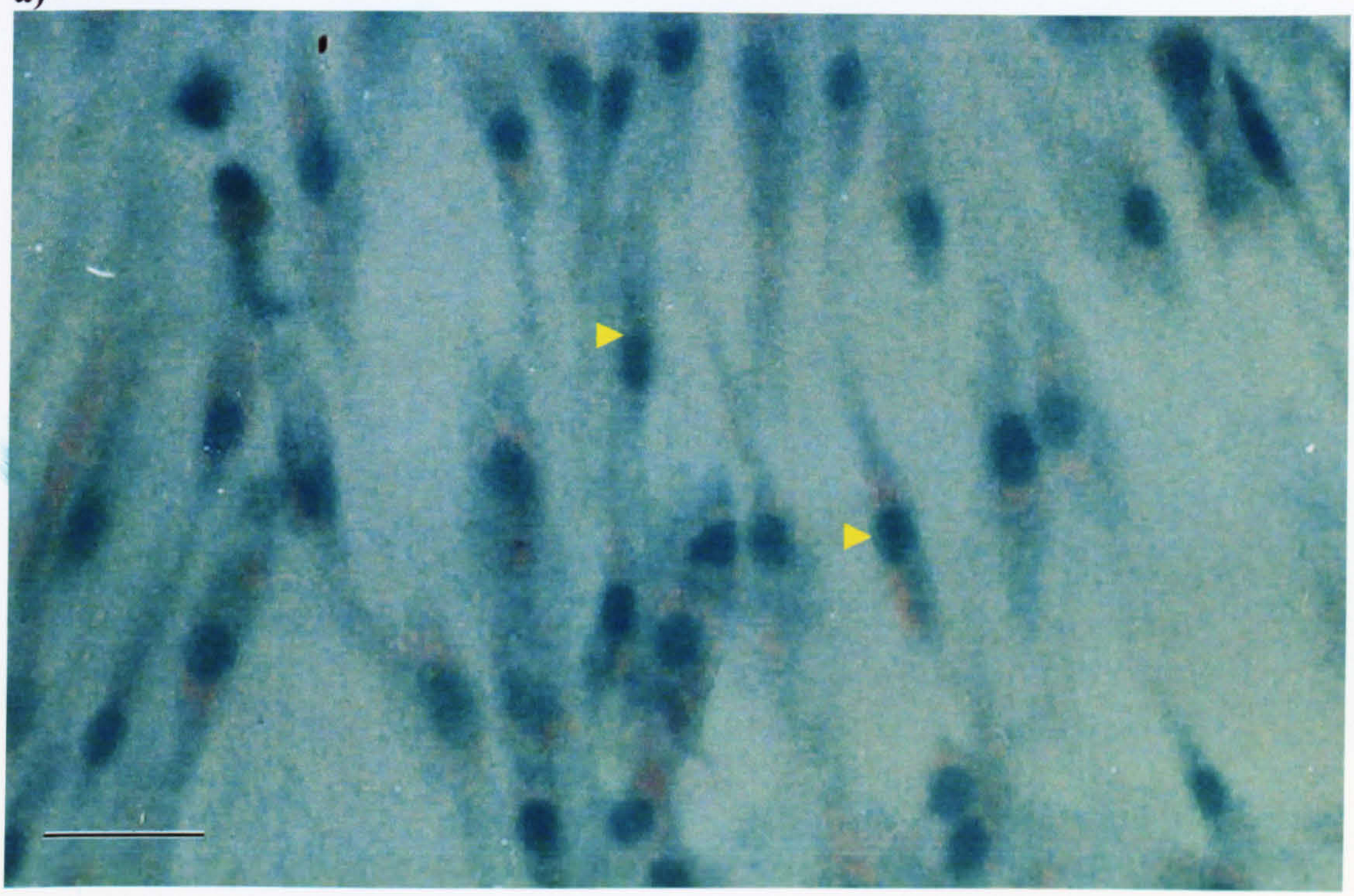
Osteoblasts are known to produce ALP, which is bound to both the nuclear and cell membranes, as well as being present in the cytoplasm. Napthol-AS-B1 and fast red violet is a histological stain for ALP, hence the occurrence of ALP within the cells was

observed as a marker of osteoblastic differentiation. The results from the 5 patients' marrow cells were the same. As seen in figure 1.3, there was minimal pink discolouration of the cytoplasm of the marrow-isolated cells cultured in standard medium.

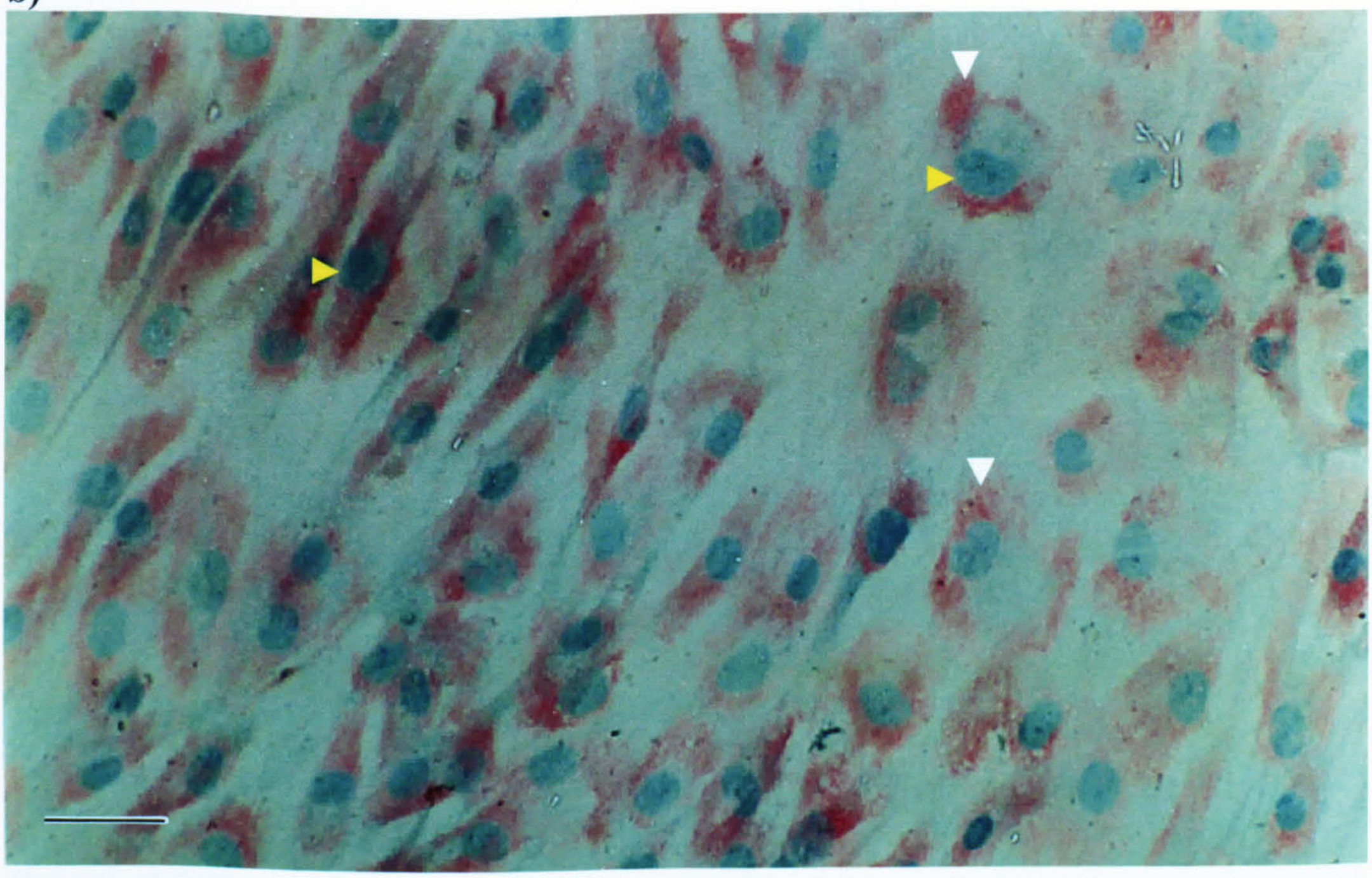
In contrast to this, the cells cultured with OS stained positively for ALP after 7 days, as seen by the red stain (figure 1.3b). After 14 days in osteogenic culture, the confluent cells continued to stain for ALP (figure 1.3c), however the intensity of the stain remained less than the osteosarcoma cell control and the occasional cell expressed significantly less ALP (figure 1.3d).

Figure 1. 3: Napthol-AS-B1 and fast red violet stain after 7 days in culture conditions, a) MSCs in standard medium, bar = 50μm, b) MSCs in osteogenic medium after 7 days, bar =50μm, c) MSCs in osteogenic medium after 14 days, bar =50μm & d) Saos-2 cells after 7 days in culture, bar =50μm, ALP stained red (white arrows), nucleus blue (yellow arrows).

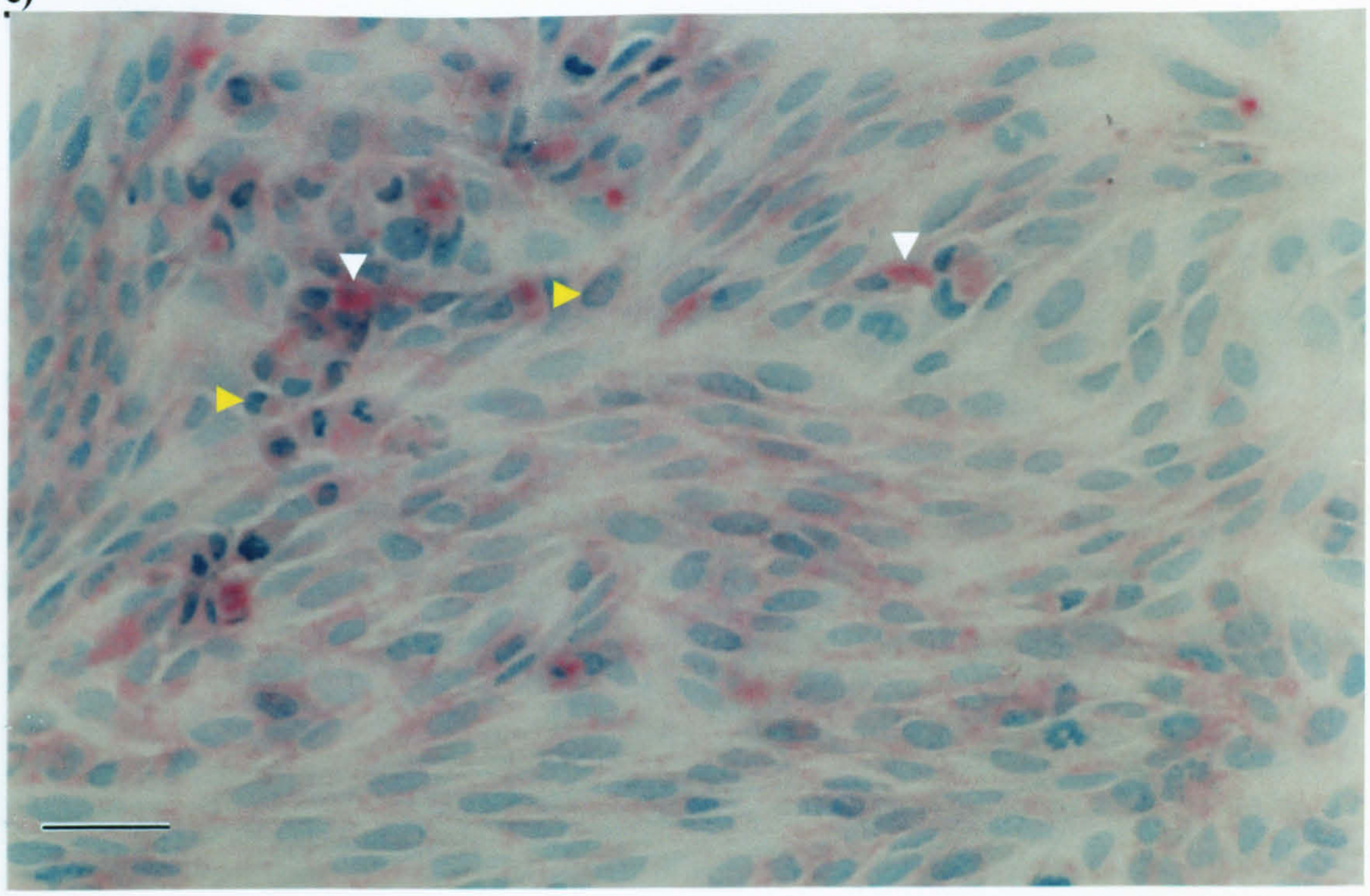
a)



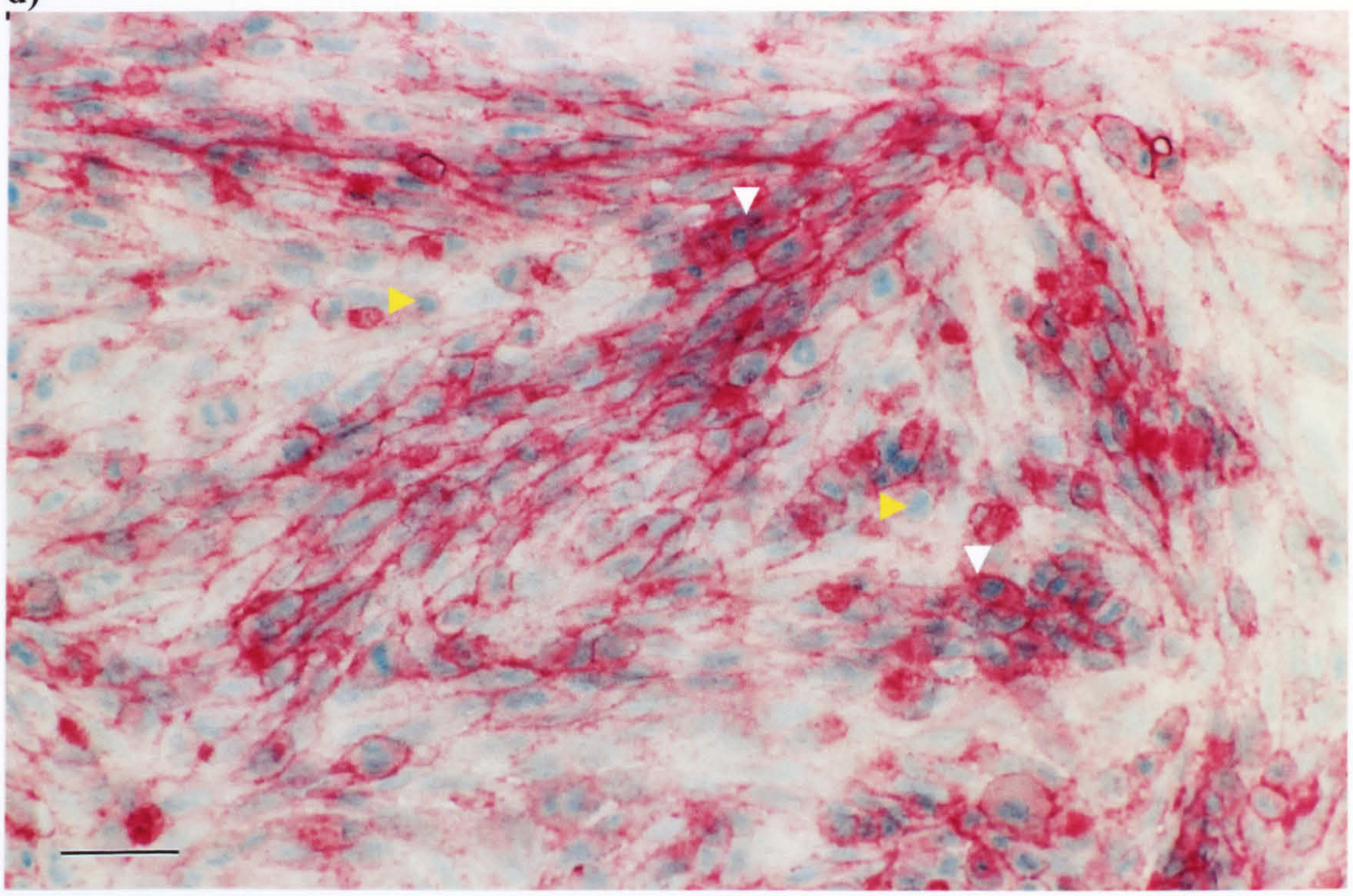
b)



c)



d)



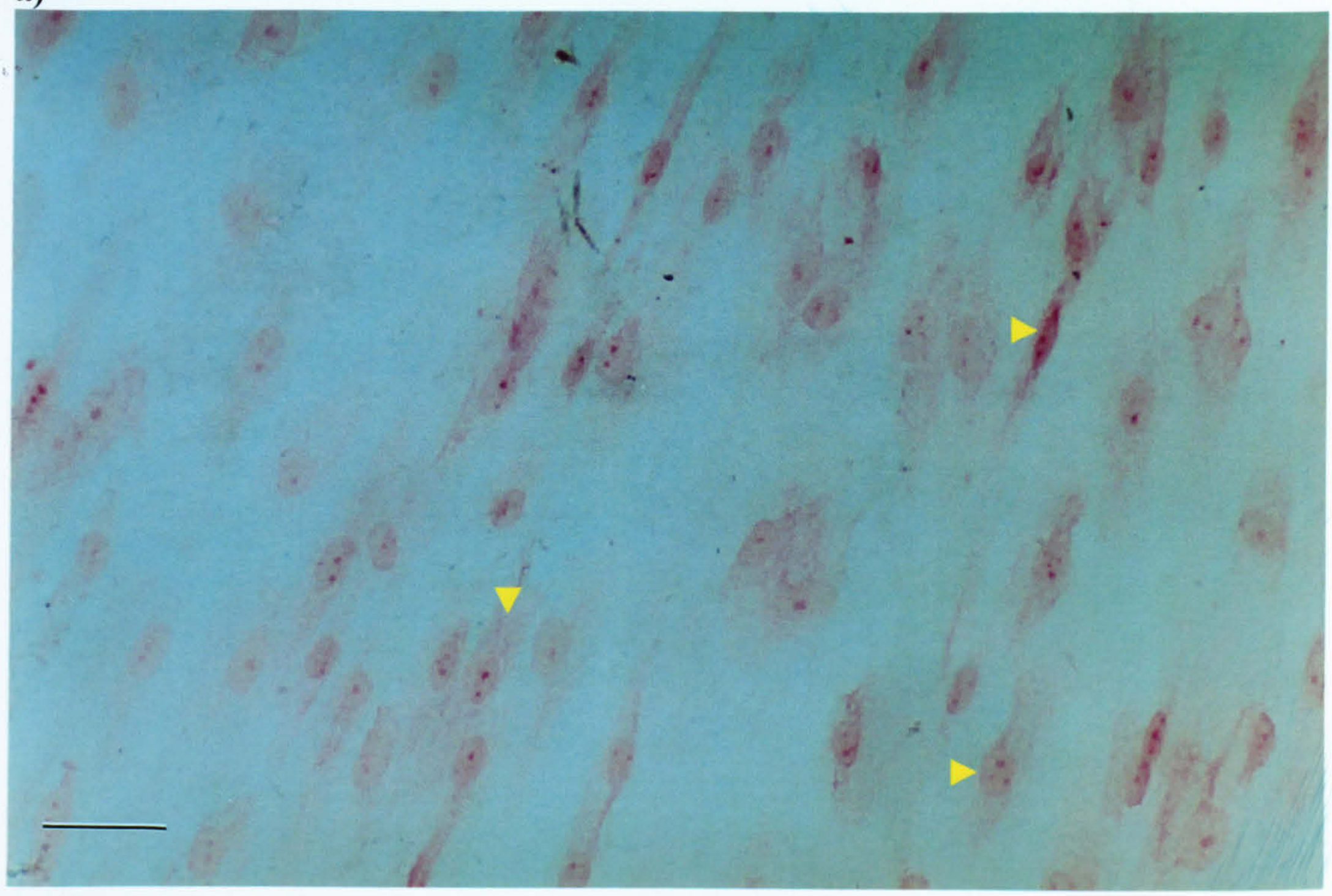
2.3.2.2 Osteoblastic differentiation – Von Kossa Histology stain

Von Kossa stain was used as a marker of calcification in the cell cultures, as an indication of extracellular mineralisation. For each of the marrow samples there was no calcium staining when the cells had been cultured for 28 days in standard medium (see figure 1.4a).

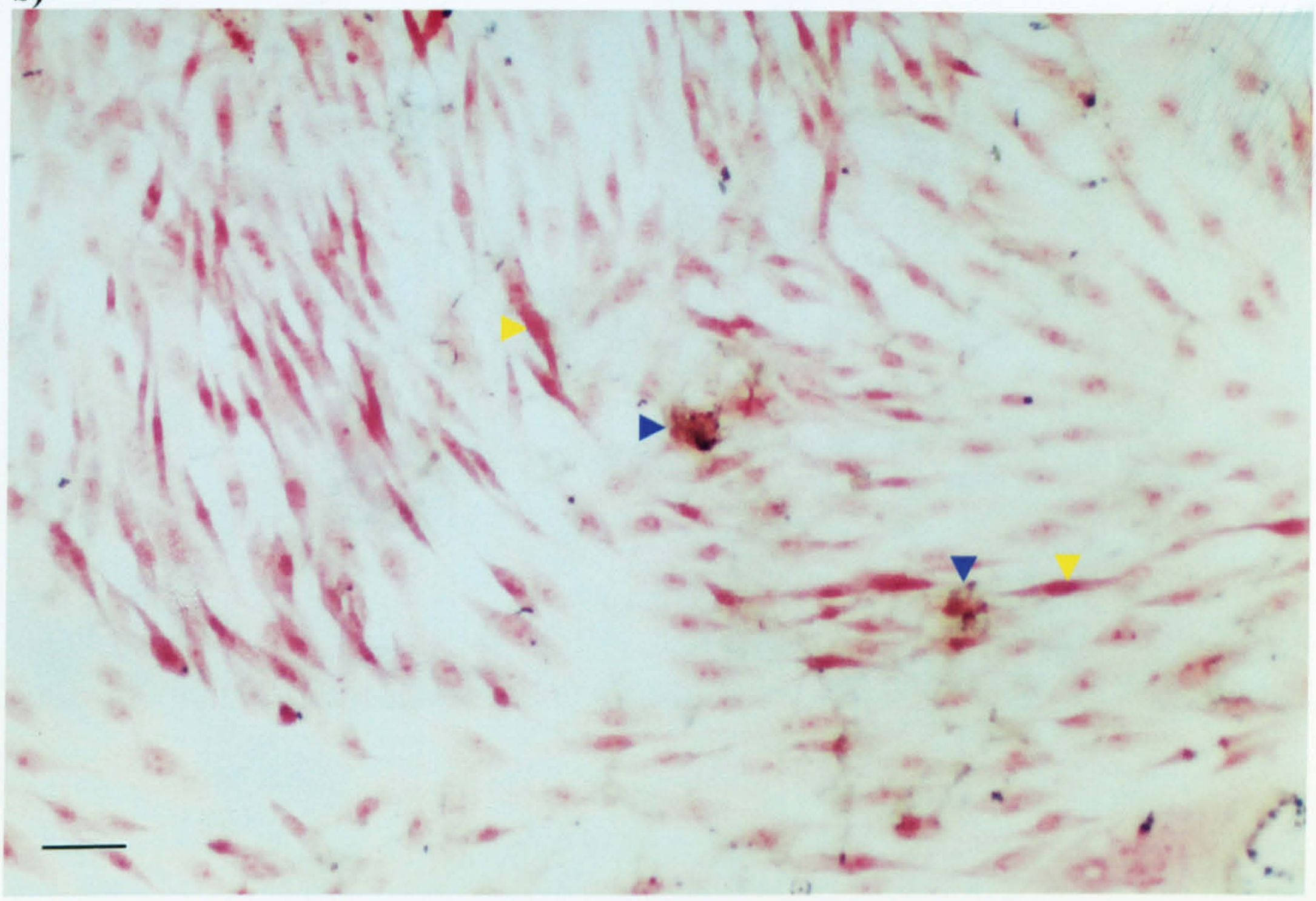
However, when the cells were cultured in osteogenic medium for 21 days there was evidence of extracellular calcium. These areas increased after 28 days in culture, although the positive osteosarcoma control produces larger areas of mineralisation (see figure 1.4c,d).

Figure 1. 4: Von Kossa stain of marrow isolated cells in culture, a) in standard culture after 28 days, bar = 50μm b) in osteogenic culture after 21 days, bar =50μm c) in osteogenic culture after 28 days bar =50μm and d) osteosarcoma cell control after 28 days, bar =50μm calcium stained black (blue arrows), cells stained red (yellow arrows).

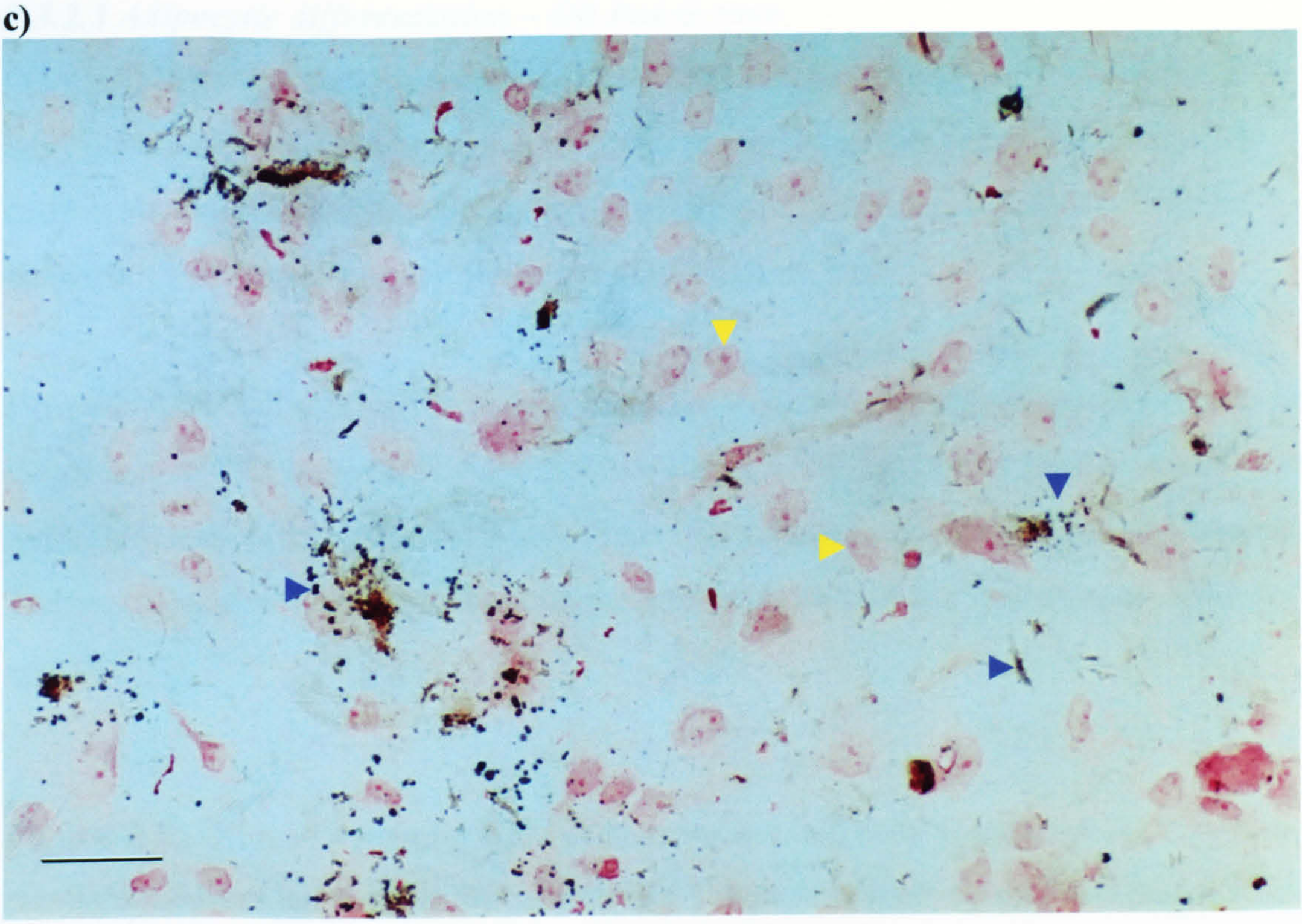
a)



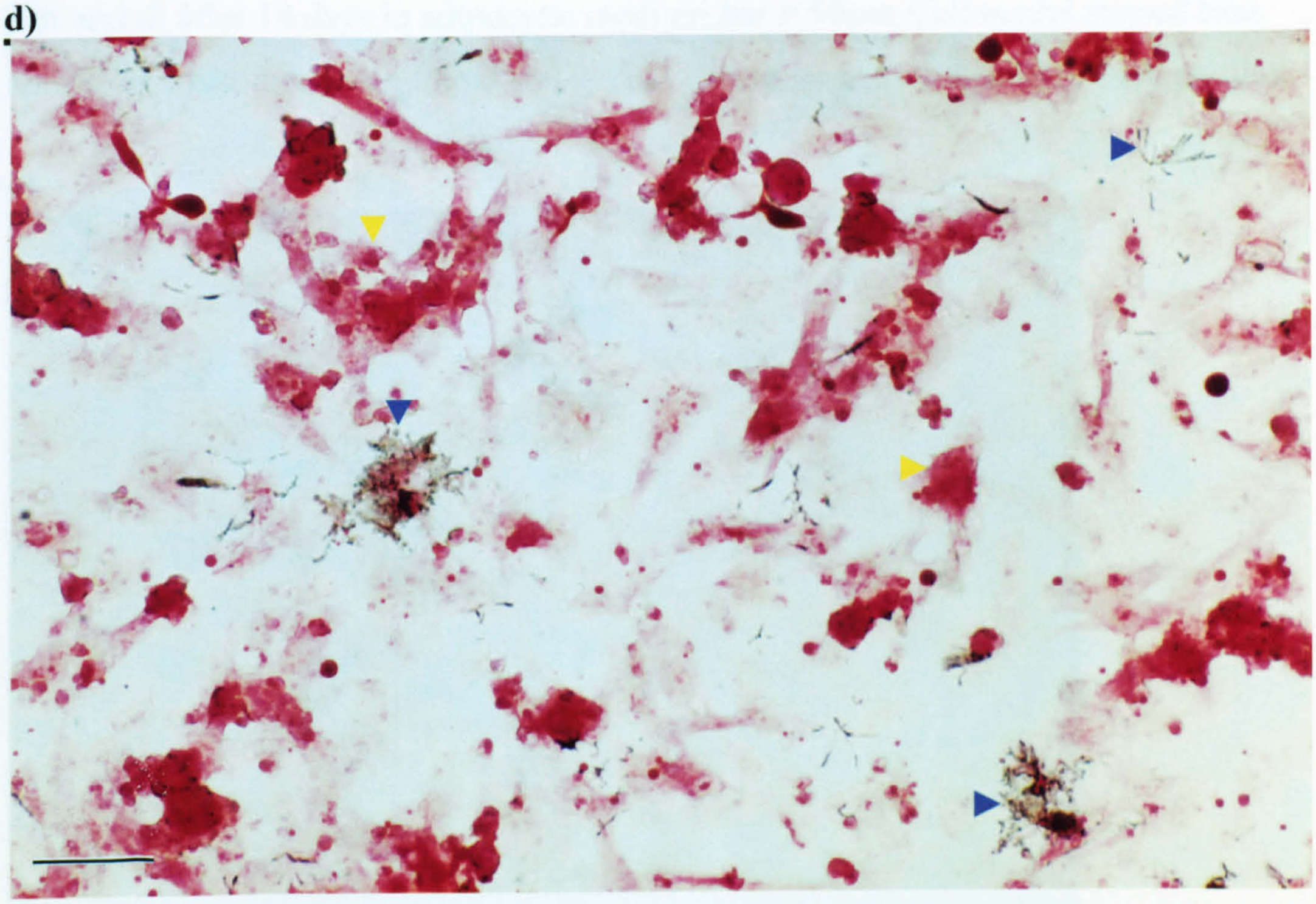
b)



c)



d)



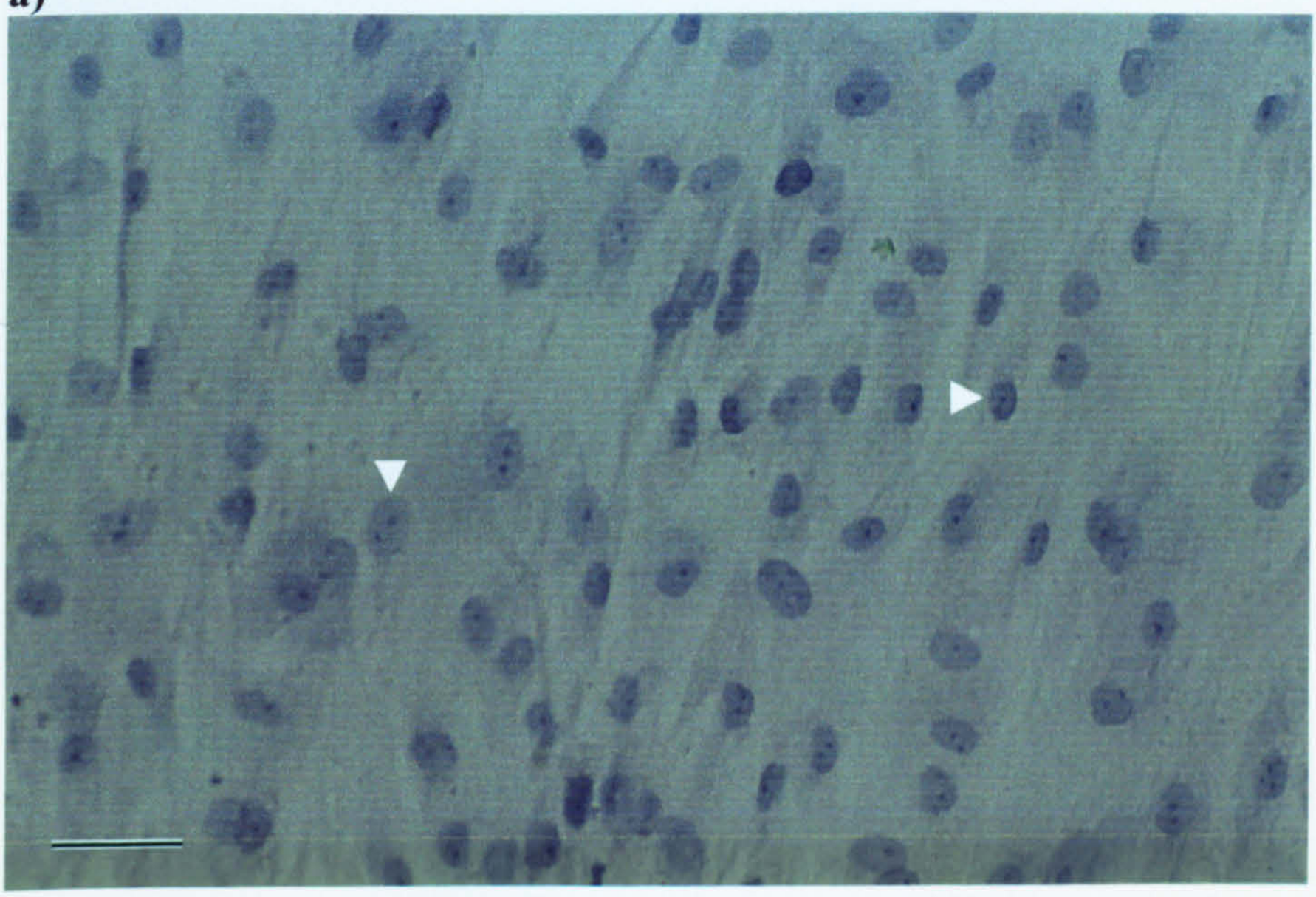
2.3.2.3 Adipocytic differentiation – Oil Red O stain

Oil red O histology stain was used to detect lipid within marrow-isolated cells after 7 days in culture with adipocytic supplements, as compared to standard medium. The cells in standard culture conditions were again noted to be long and spindle-like in shape, with minimal lipid within the cells (see figure 1.5a).

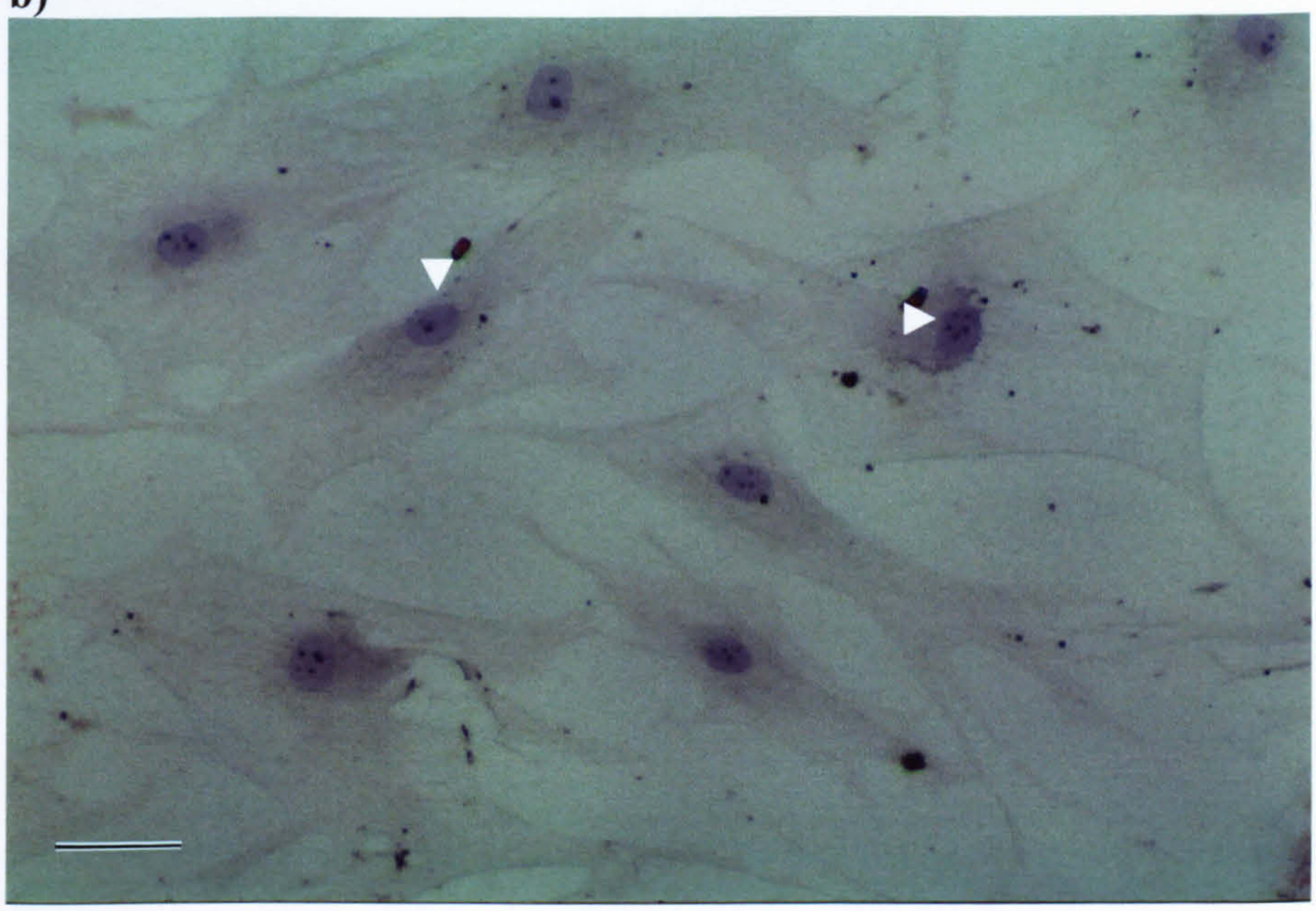
However, cells cultured in the presence of adipocytic supplements were observed to be larger in size with increased cytoplasm (see figure 1.5b). By day 14 lipid droplets were frequency seen within the cytoplasm of the cells cultured with adipocytic supplements. These results were consistent for cells isolated from each of the four marrow aspirates tested.

Figure 1. 5: Oil red O stain for lipid in marrow-isolated cells a) cultured for 7 days in standard medium, bar = 50μm, b) cultured for 7 days in adipocytic medium, bar = 50μm, c) after 14 days in adipocytic medium, showing lipid droplets within the cell, bar = 50 μm and d) after 14 days in adipocytic medium, bar = 50μm. Cell nuclei stained blue shown by white arrows and lipid stained red shown by yellow arrows, where present.

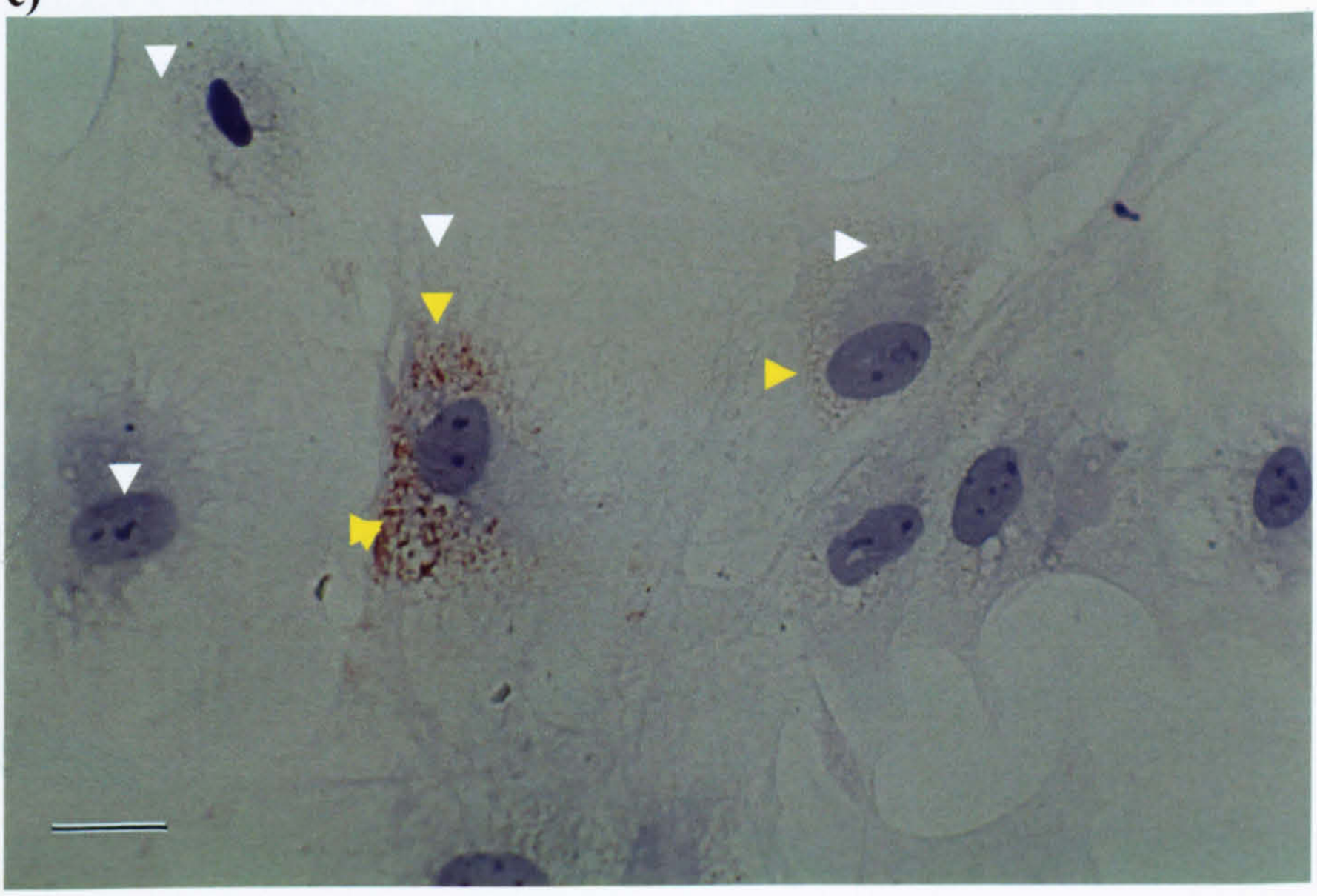
a)



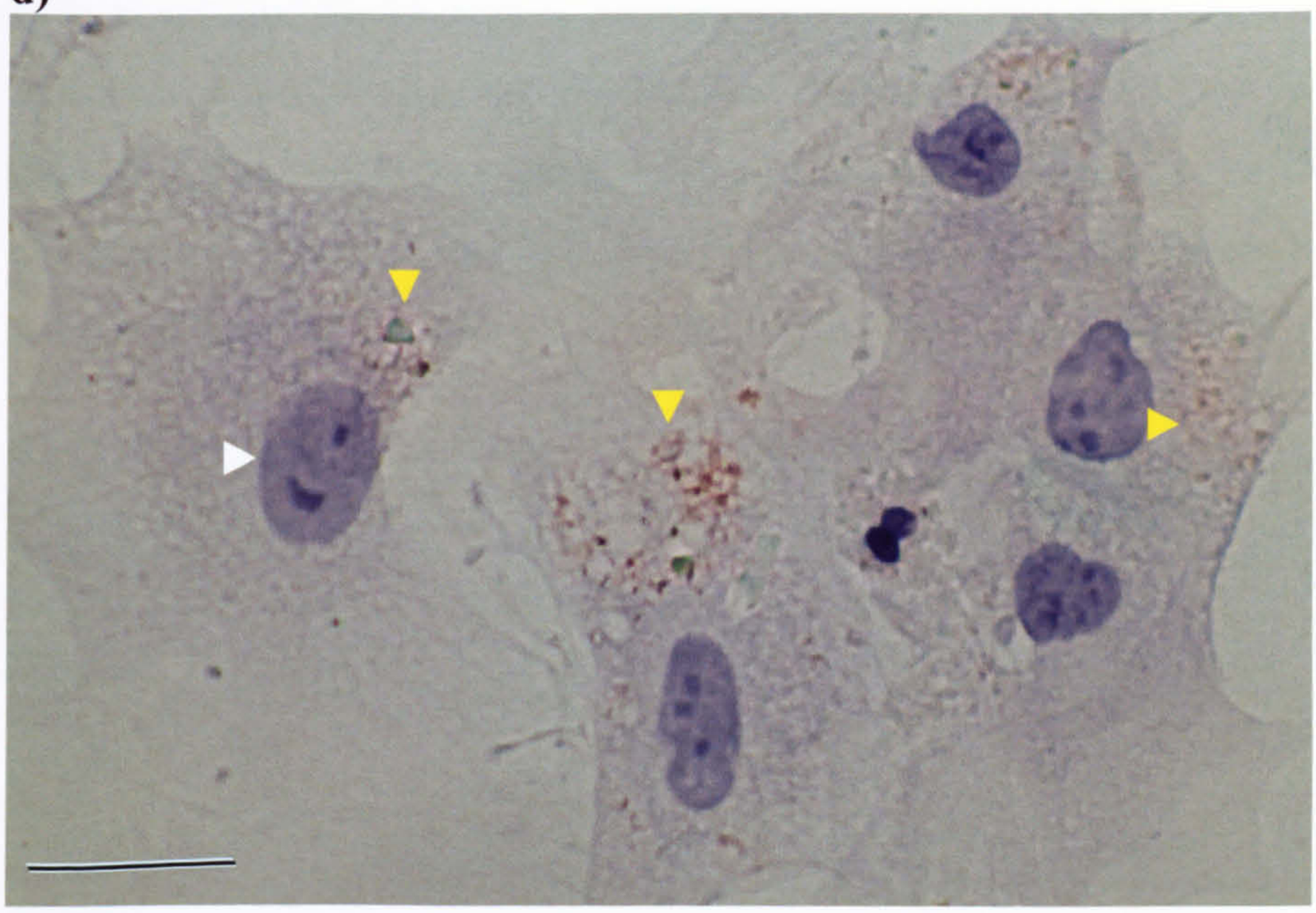
b)



c)



d)



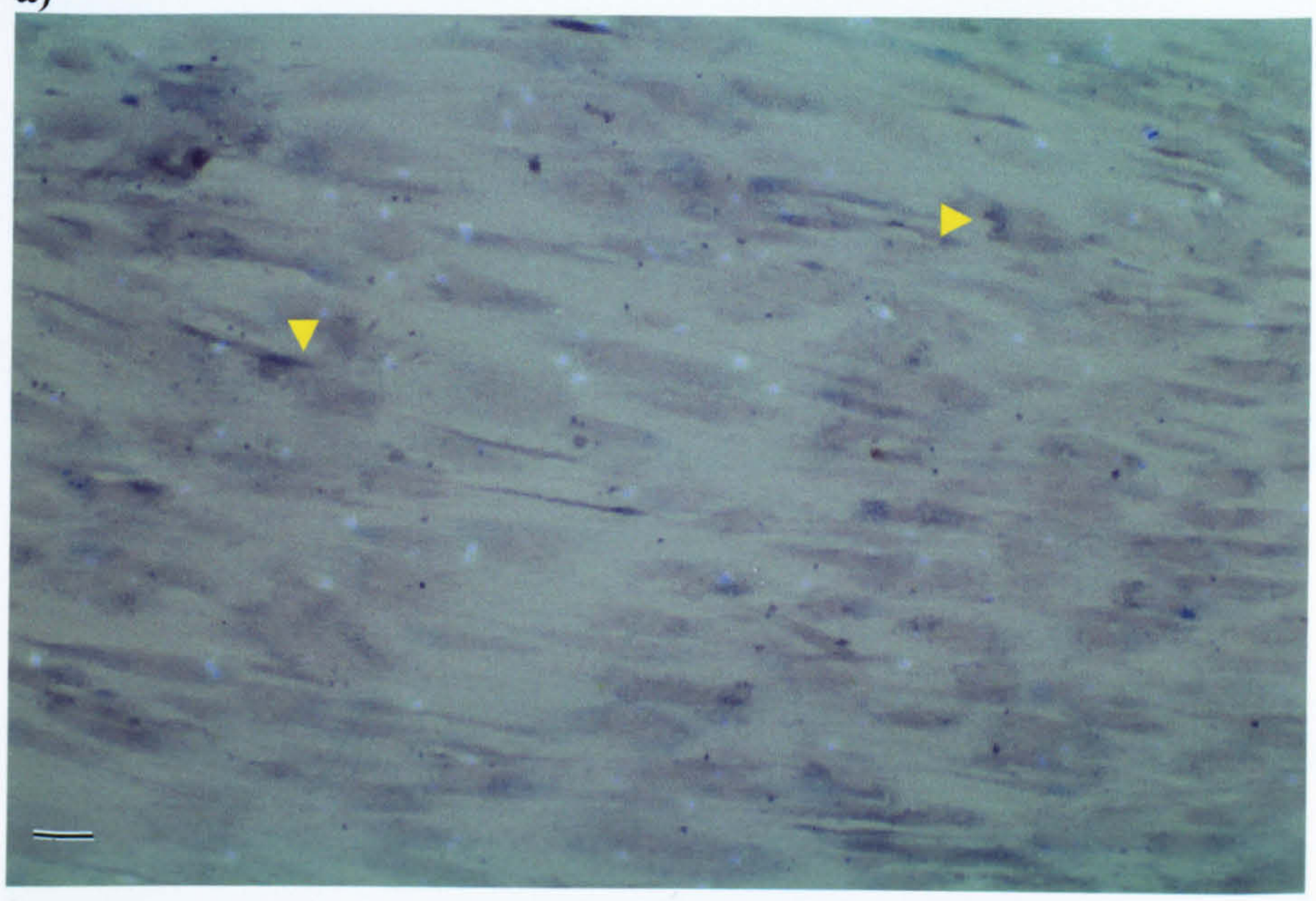
2.3.2.4 Chondrocytic differentiation – Alcian blue/Sirius red stain

Alcian blue/sirius red was used to stain collagen type I and II organised extracellular matrix, which polarises red/green under polarised light. No collagen was found on the slides of marrow-isolated cells in standard or osteoblastic medium and a likewise negative result was gained from the bovine chondrocyte controls (see figure 1.6a,b,c).

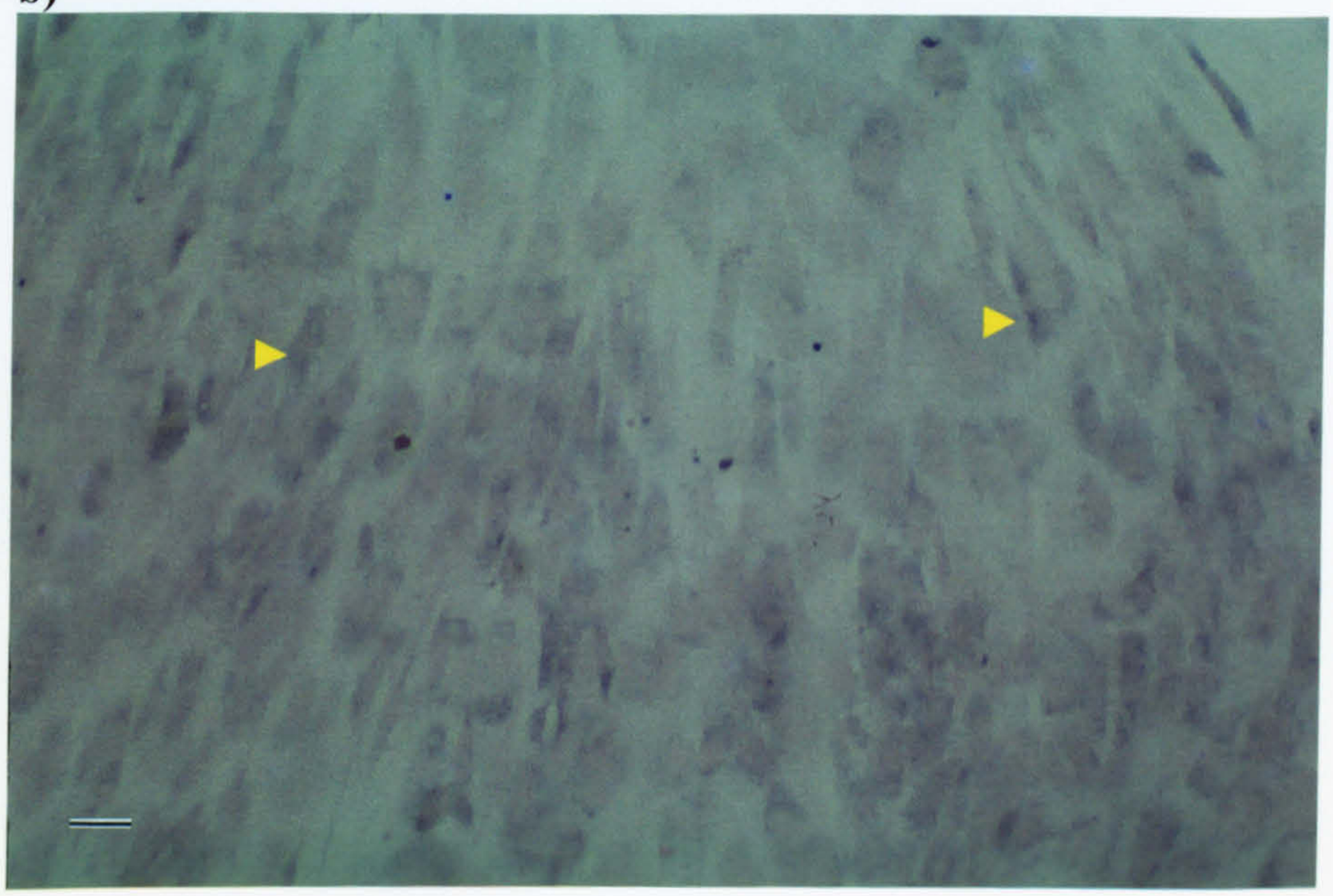
However, when the marrow isolated cells had been cultured in chondrocytic medium for 14 days, the cells were observed to clump together and collagen matrix was seen surrounding the clumps. This collagen was arranged in a lattice structure, with fibres at 90° to each other thus polarising red or green respectively (see figure 1.6d,e).

Figure 1. 6: Alcian blue/sirius red stain for collagen was used for marrow-isolated cells a) cultured for 14 days in standard medium, bar =50μm b) cultured for 14 days in osteoblastic medium, bar =50μm c) bovine chondrocytes after 14 days in culture, bar = 50 μm and d) after 14 days in chondrocytic medium, bar =50μm and e) after 14 days in chondrocytic medium, bar = 50μm. Where present, collagen stained red/green under polarised light (white arrows) and cells stained blue (yellow arrows).

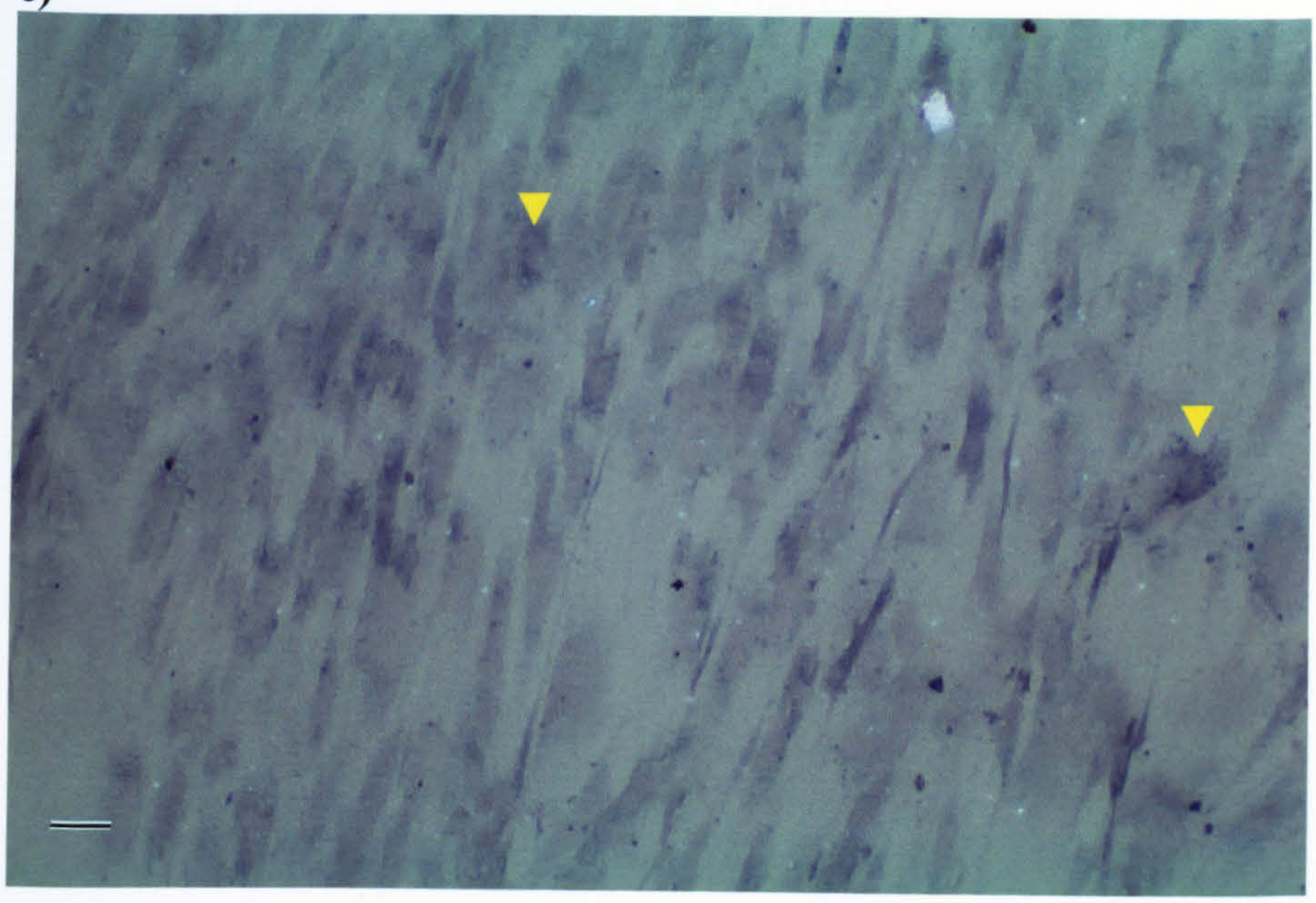
a)



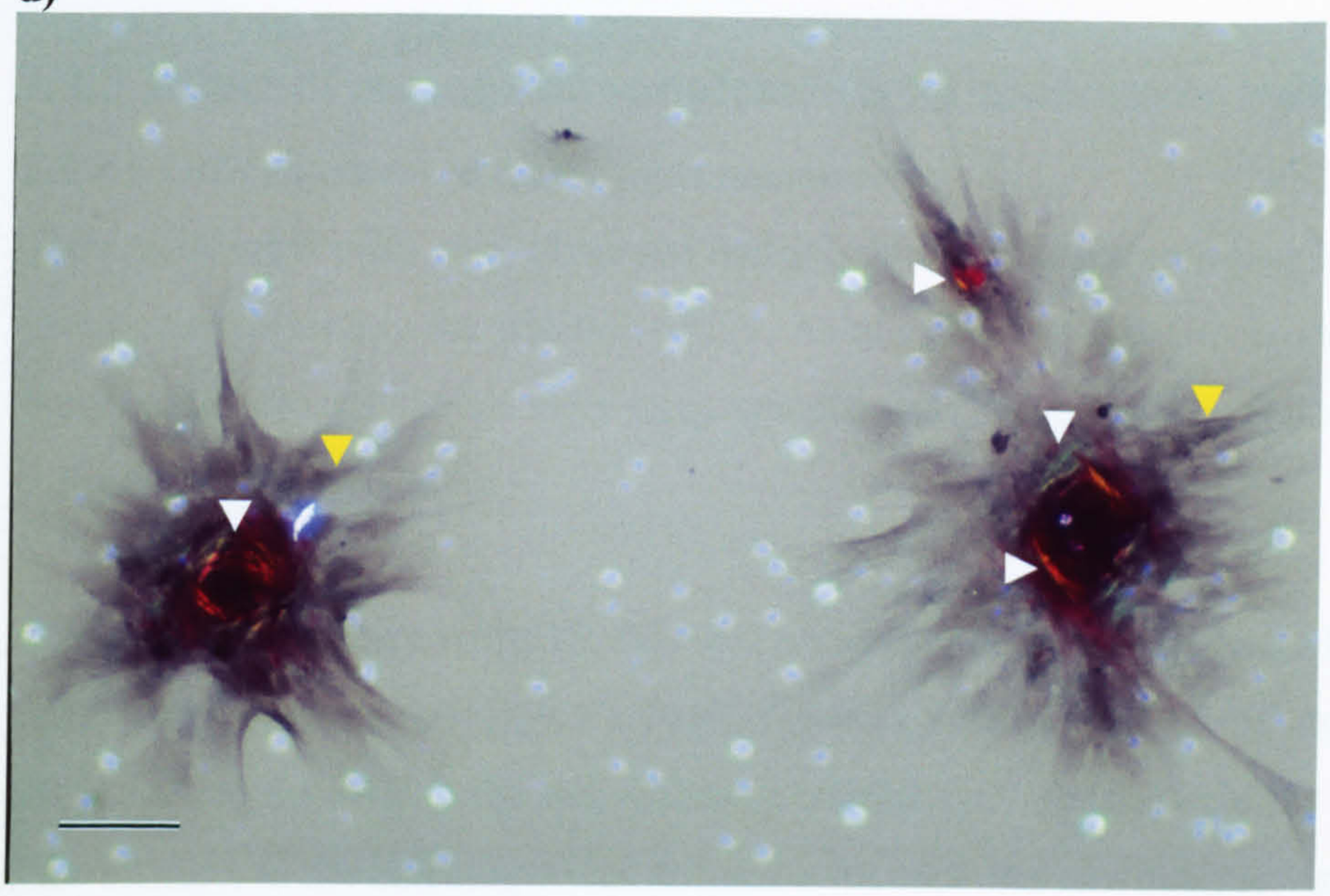
b)



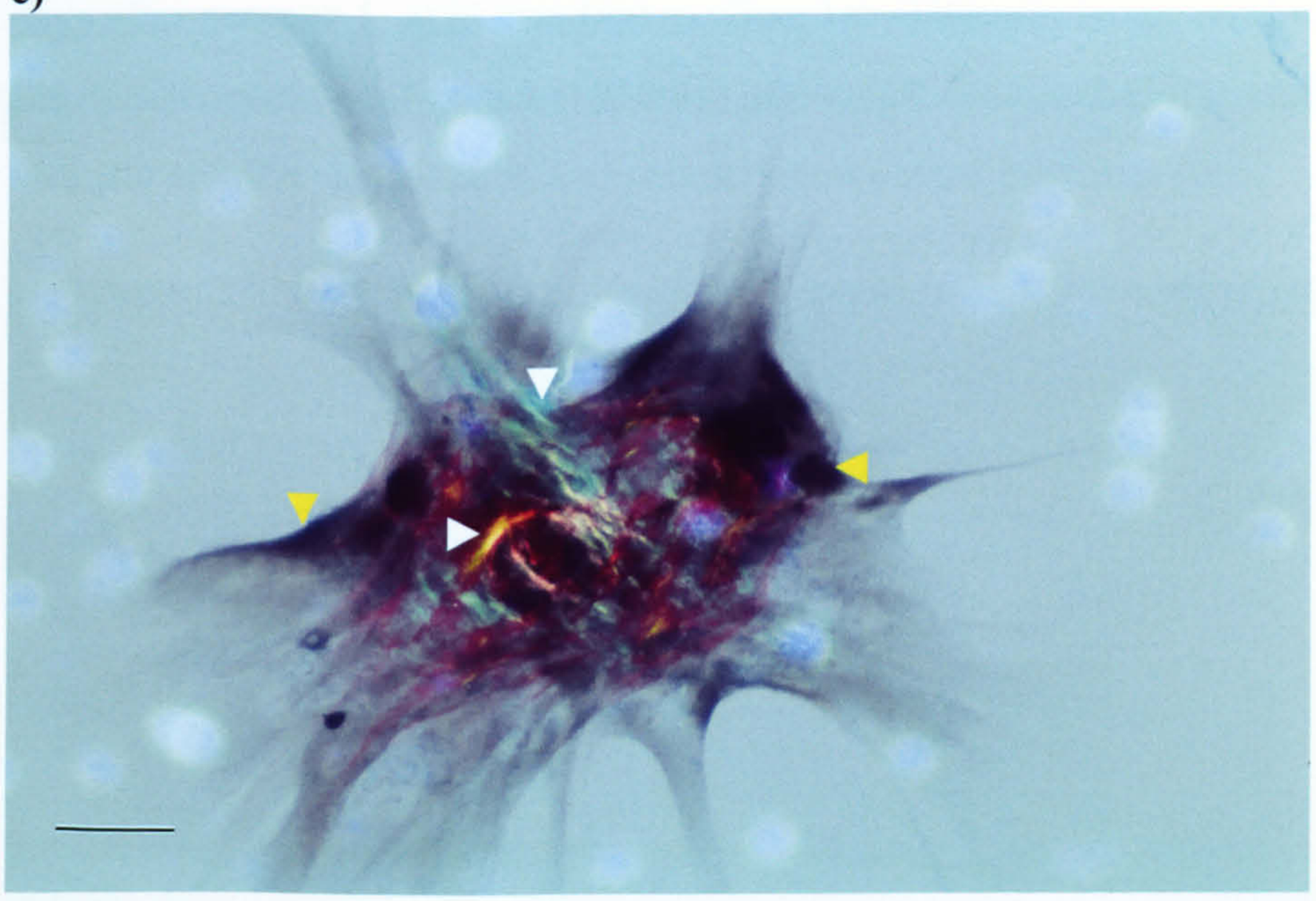
c)



d)



e)



2.3.3 Effect of osteogenic supplements on monolayer culture

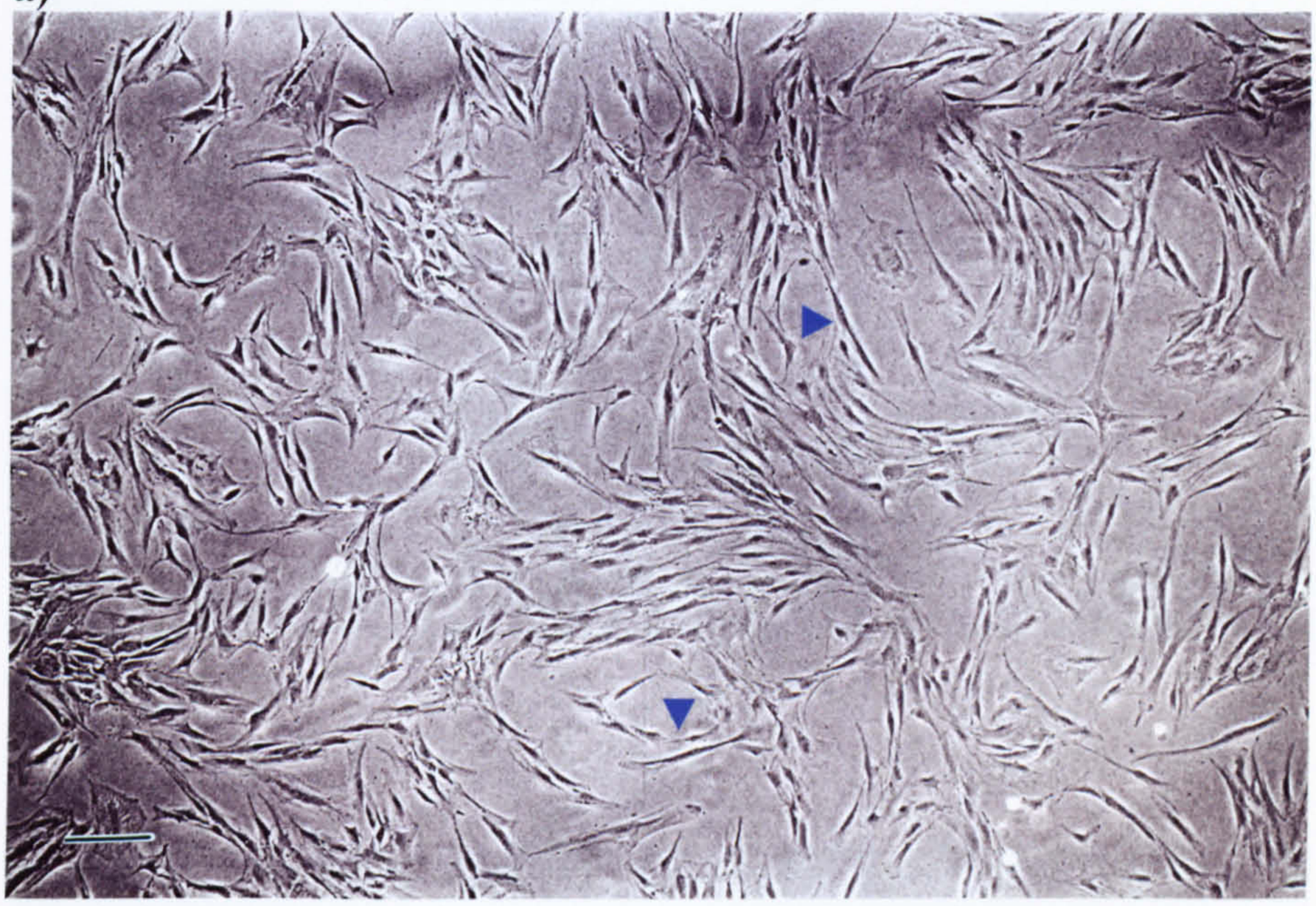
2.3.3.1 Cells as observed under light microscopy

Marrow isolated cells were observed under light microscopy growing in standard culture and following the addition of OS. As described above, the marrow cells in standard culture conditions were long, spindle cells, similar to fibroblasts in monolayer culture (see figure 1.7a,b).

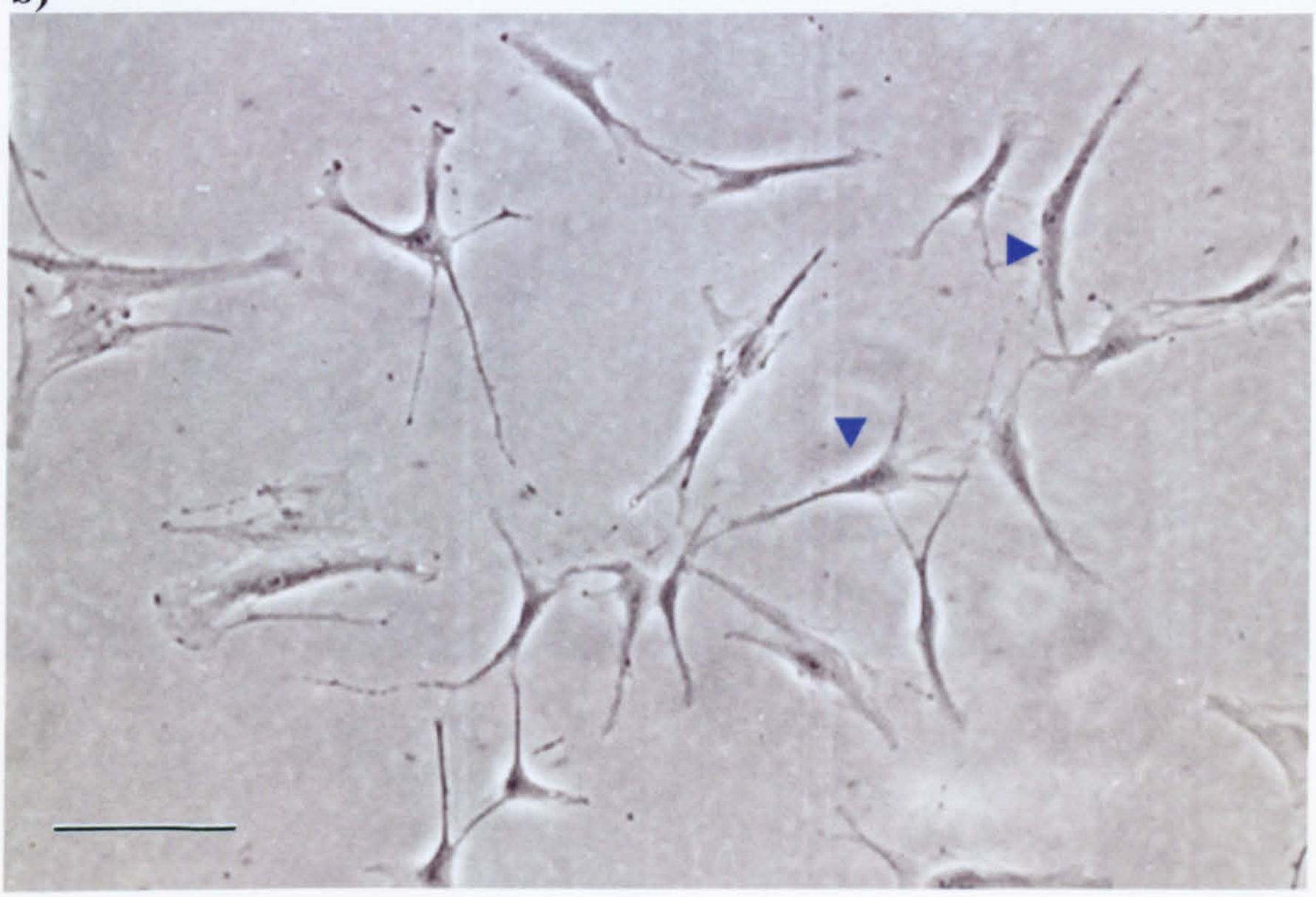
After 5 days in culture with OS, this was noticed to change as the cells became smaller and squarer in shape (see figure 1.7c,d). Figure 1.8 shows a clear difference between the shapes of the cells in the two different conditions, once the cultures were confluent. This change, in the cell shape was noticed with each marrow sample, on addition of OS to the culture conditions.

Figure 1. 7: Marrow isolated cells under light microscopy in monolayer culture, 5 days after passage 1, a) in standard medium,bar=200µmb) in standard medium, bar =200 µm c) osteogenic mediumbar=200µmd) osteogenic mediumbar=200µmBlue arrows indicate spindle shaped cells and yellow arrows show cuboidal cells.

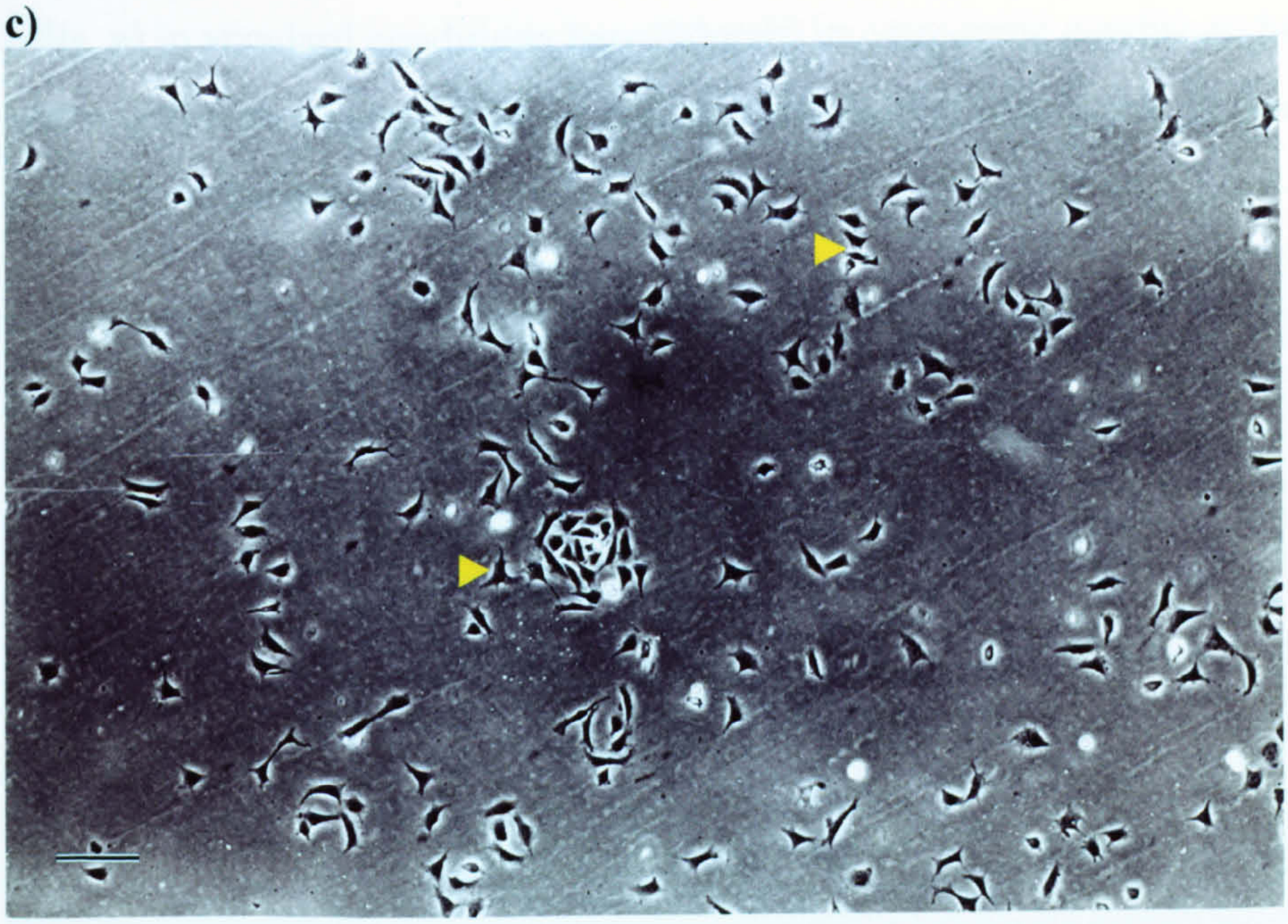
a)



b)



c)



d)

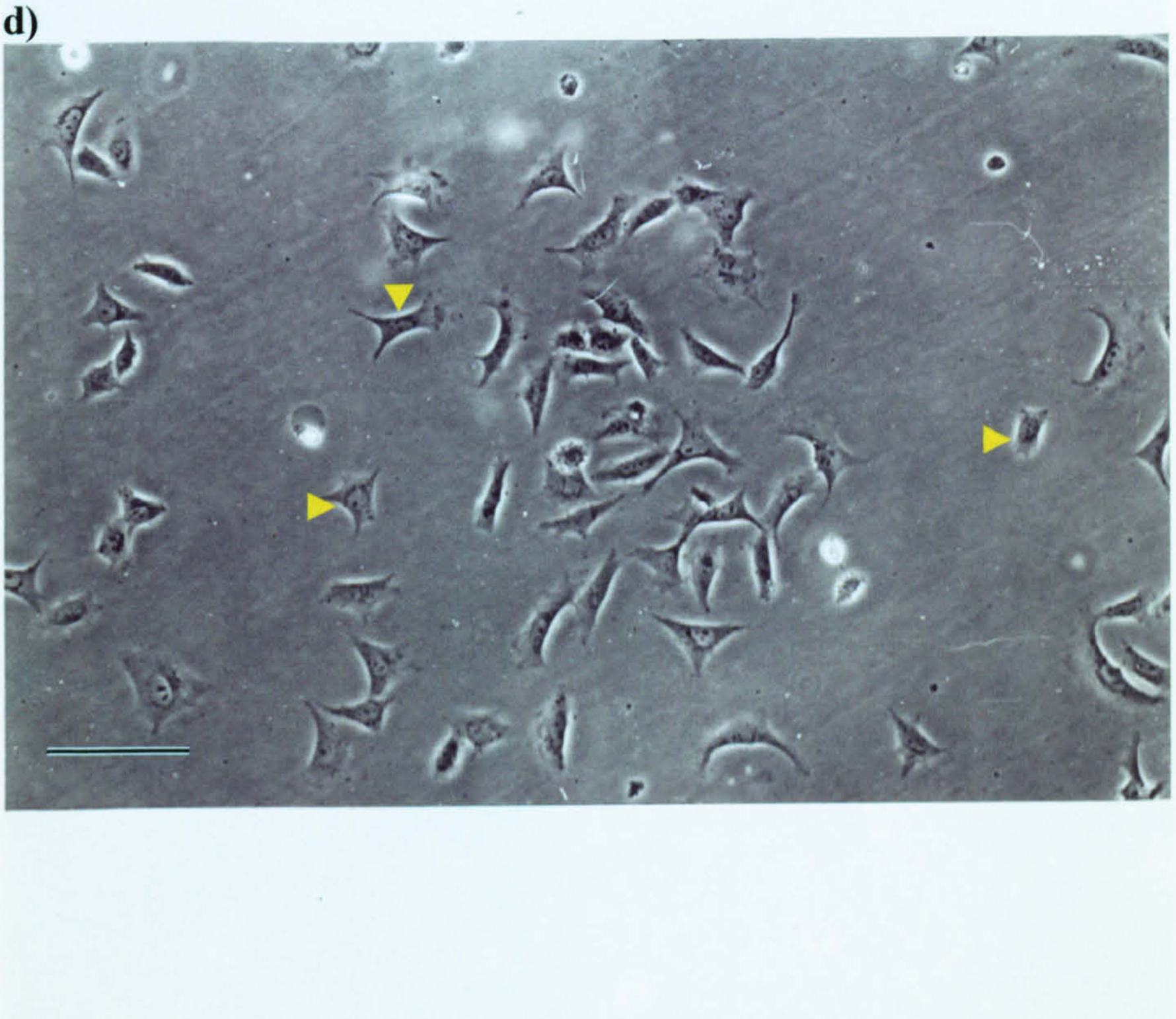
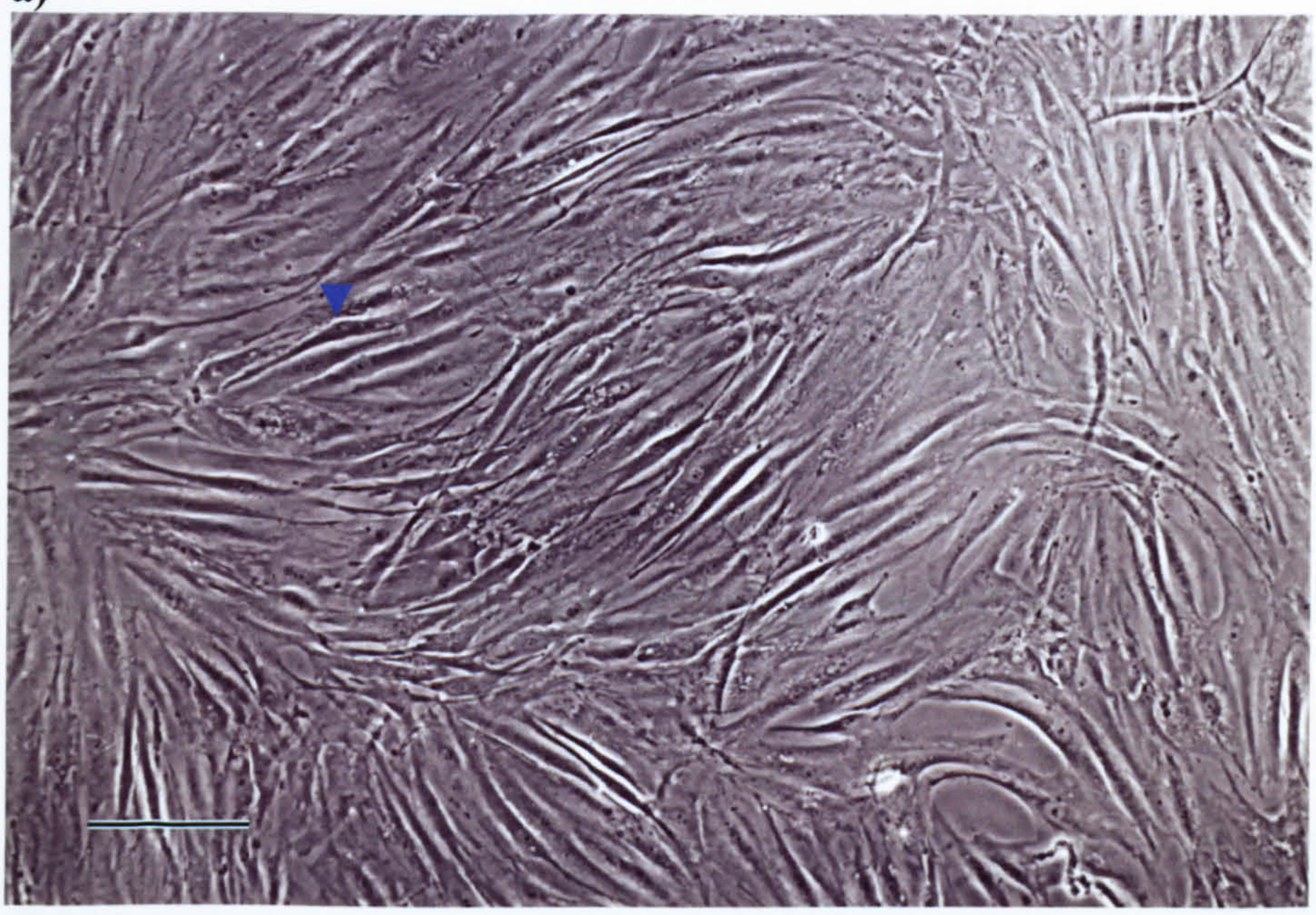
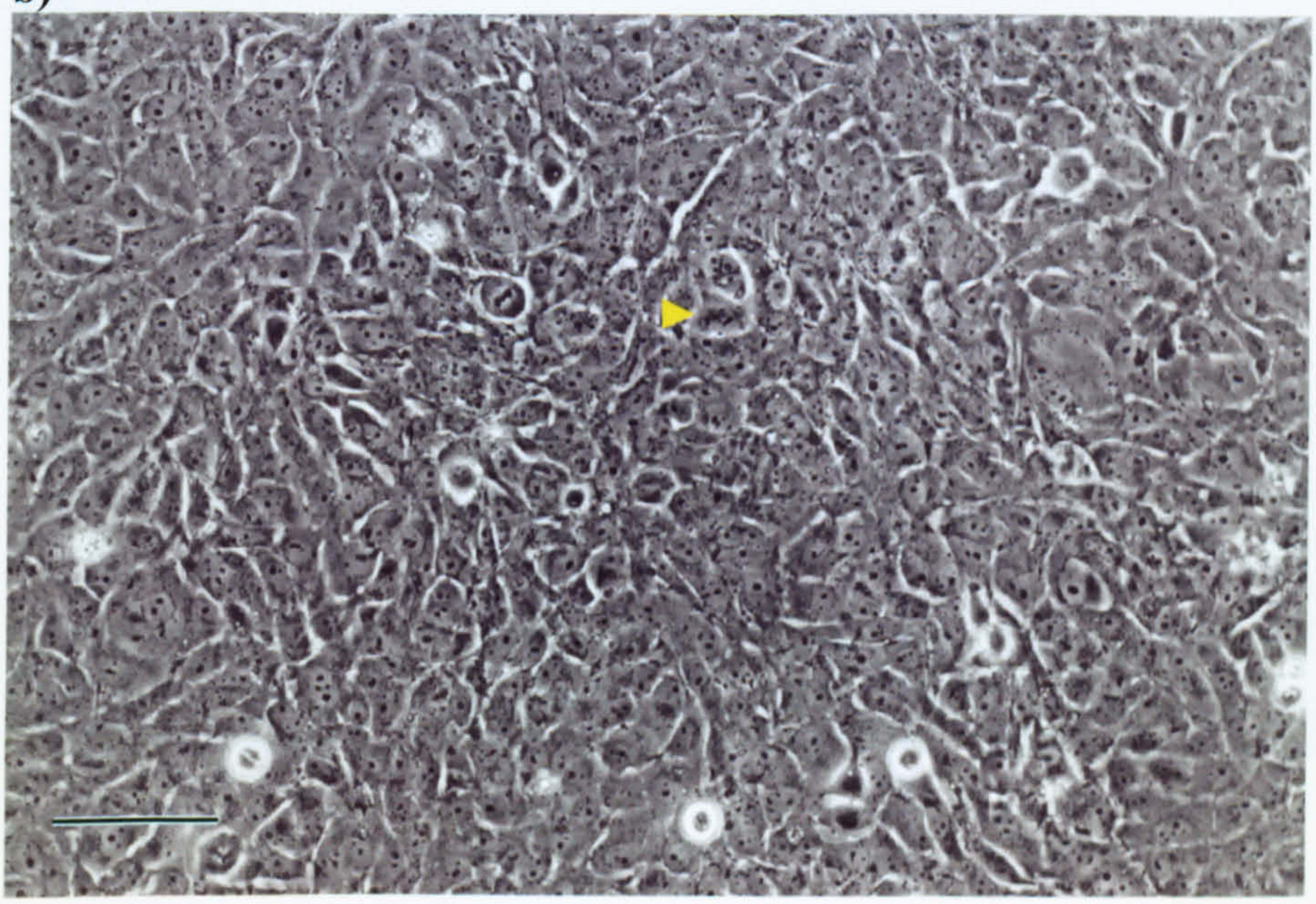


Figure 1. 8: Light microscopy pictures of confluent cultures of bone marrow isolated cells, a) in standard conditions,bar=200μmb) in osteogenic conditions,bar=200μmBlue arrow indicates a spindle shaped cell characteristic of MSCs and yellow arrow shows a cuboidal osteoblastic cell.

a)



b)



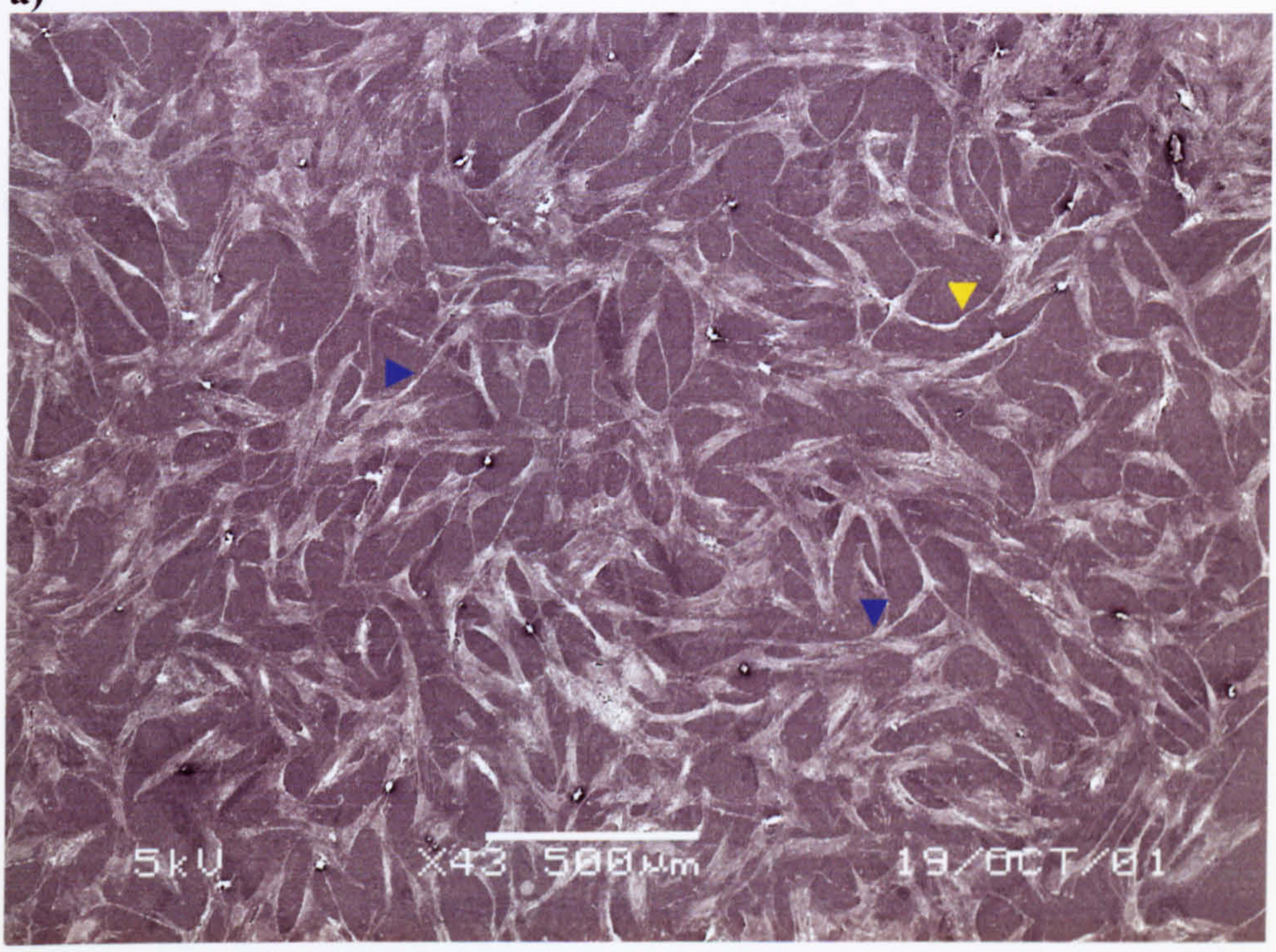
2.3.4.1 2.3.3.2 Cell morphology under Scanning Electron Microscopy (SEM)

The effect of addition of OS to the morphology of marrow cells was further observed under SEM (see figure 1.9). Cells cultured in standard medium for 24 hours were fibroblast-like in morphology and this persisted during the 7-day culture period.

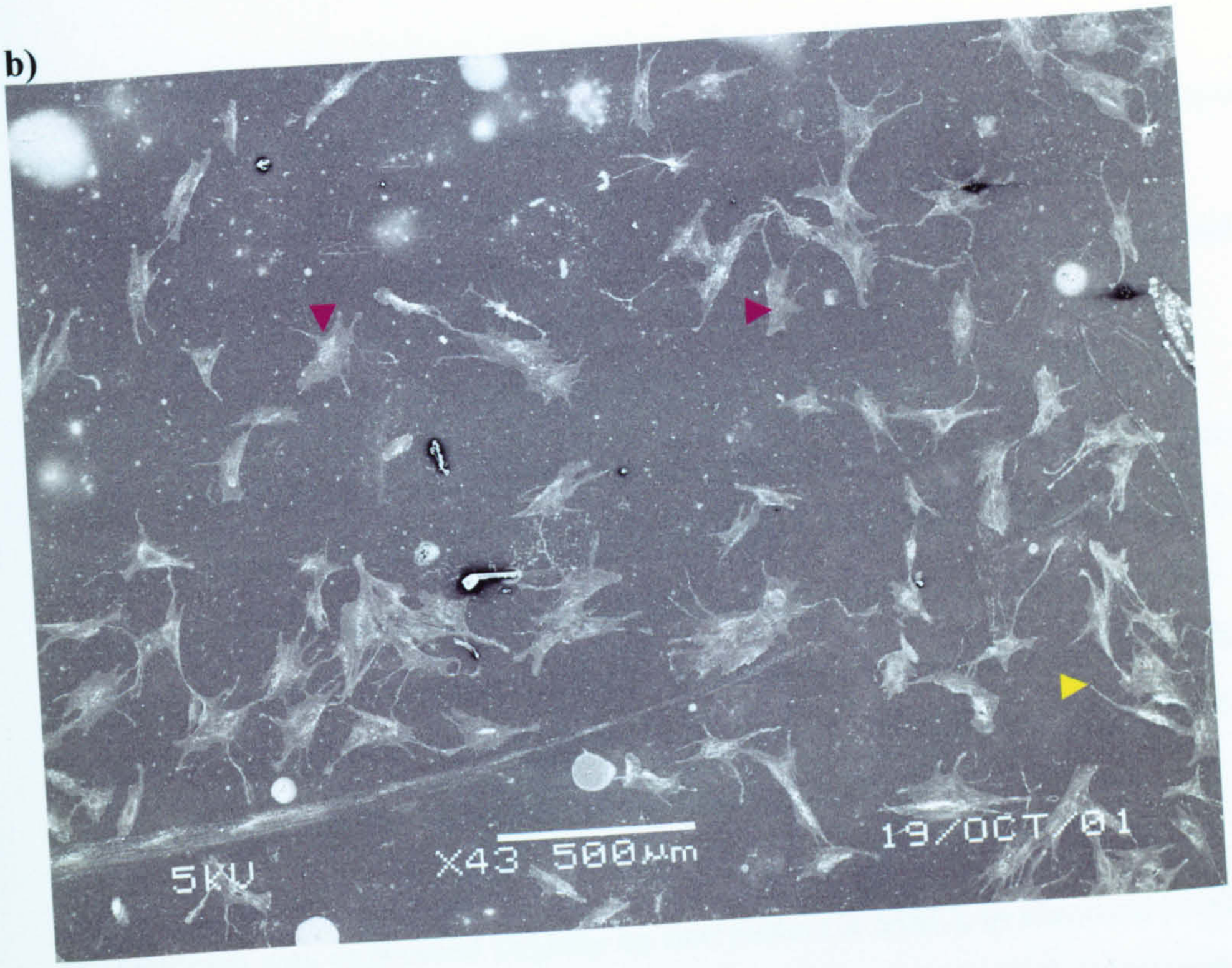
In contrast to this, cells stimulated with OS became square in shape, producing multiple processes attaching to the therminox and these changes increased over 7 days.

Figure 1. 9: This figure shows cells cultured on therminox under SEM, a) standard medium for 24 hours, b) OS for 24 hours and c) OS for 7 days showing squarer osteoblastic-like cells. The blue arrows indicate fibroblast-like morphology of MSCs, pink arrows show cuboidal cells and yellow arrows show cell processes attaching to the therminox surface. Magnification is indicated by bars on figures.

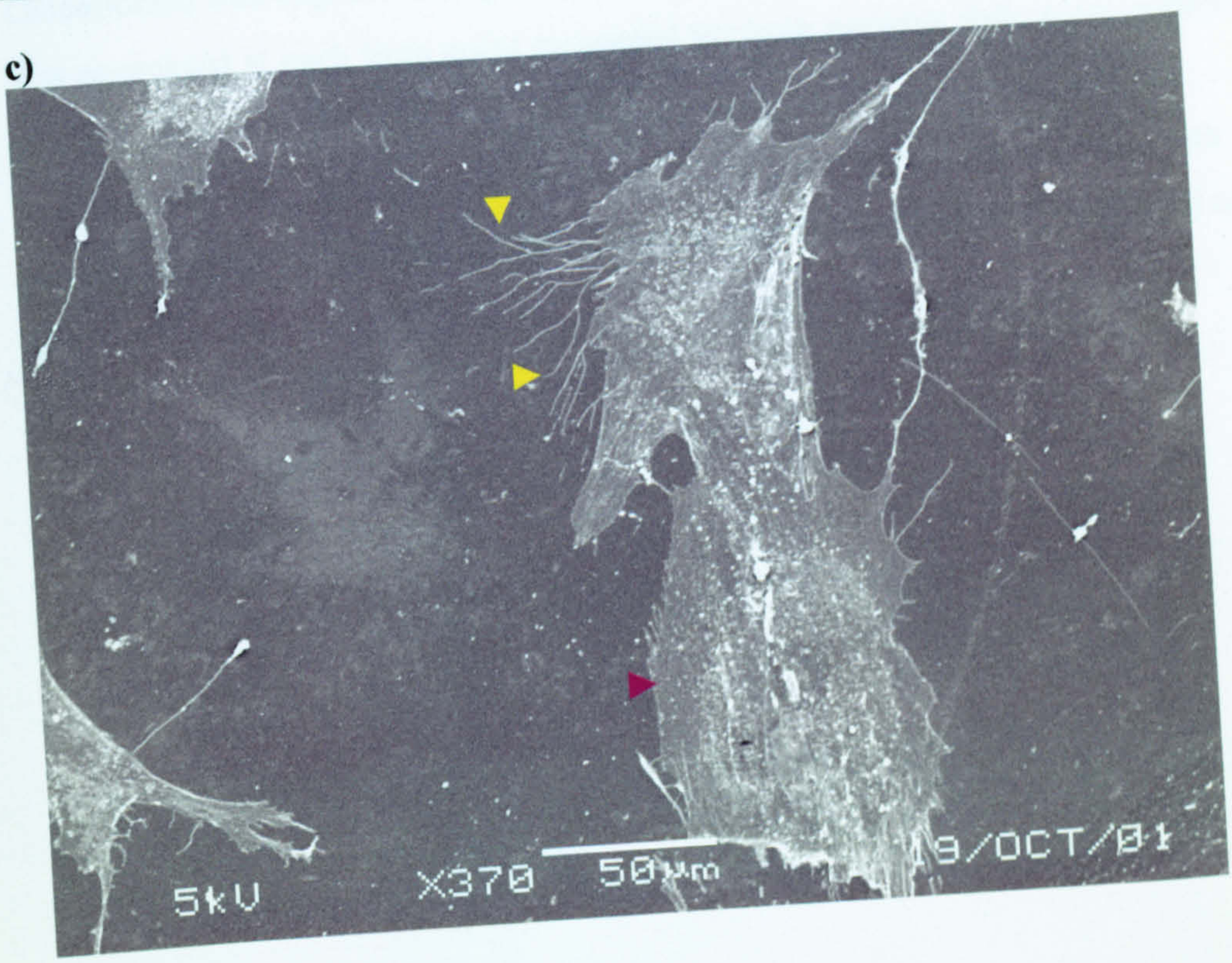
a)



b)



c)



2.3.3.3 Results for Gene expression

Gene expression of bone marrow derived cells in standard medium was compared with osteogenic supplemented medium over a 7-day culture period. GAPDH, a housekeeping gene normally expressed by human cells, was expressed by every sample tested.

Cbfa-1 was expressed by the MSCs in standard culture for all three of the patients tested. To assess the relative expression of Cbfa-1 over this culture period, the intensity of cDNA bands on the electrophoresis gel of Cbfa-1 was compared with GAPDH, for each sample. For all three patients the expression of Cbfa-1 relative to GAPDH increased after the addition of OS, peaking after 3 days and decreasing to a minimal level after 7 days in culture (see figure 1.10).

Osteopontin was expressed slightly by one patient's MSCs in standard medium, but was expressed at each time point measured for both patients following culture in OS.

In contrast to this, cells in standard medium did not express osteocalcin, with osteocalcin first being detected after 1 day in OS for one patient sample and after 3 days for the other (see figure 1.11).

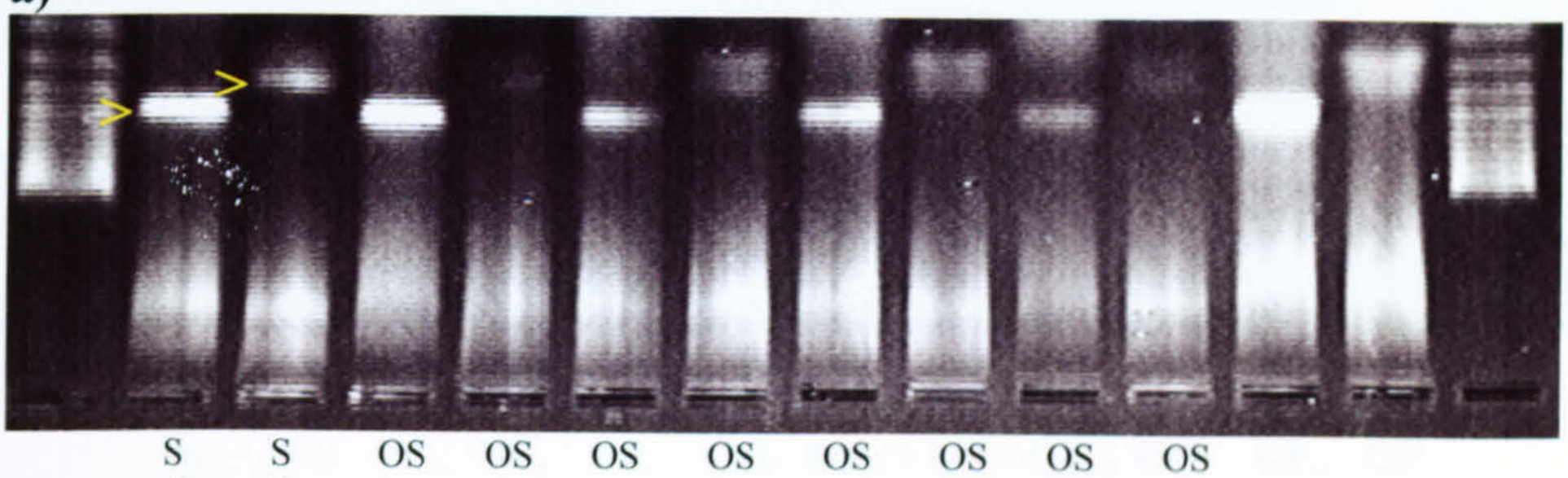
Figure 1. 10: Pictures of electrophoresis gels showing the effect of the addition of OS to the expression of Cbfa-1 and GAPDH by bone marrow isolated cells over 7 days. For each gel, yellow arrows indicate gene bands of Cbfa-1 and GAPDH:

a) Patient 1: day 0 in standard medium culture (S), and day (d) 1, 3, 5, 7 after culture with OS for the gene expression of Cbfa-1 (C) and GAPDH (G); with Saos-2 osteosarcoma cell line as positive control for osteoblasts,

b) Patient 2: Cbfa-1 (wells 2-6) & GAPDH (wells 8-12) gene expression, comparing MSCs cultured in OS with the control (S) in standard medium,

c) Patient 3: GAPDH (wells 2-7) & Cbfa-1 (wells 8-13) gene expression, comparing MSCs cultured in OS with the control (S).

a)



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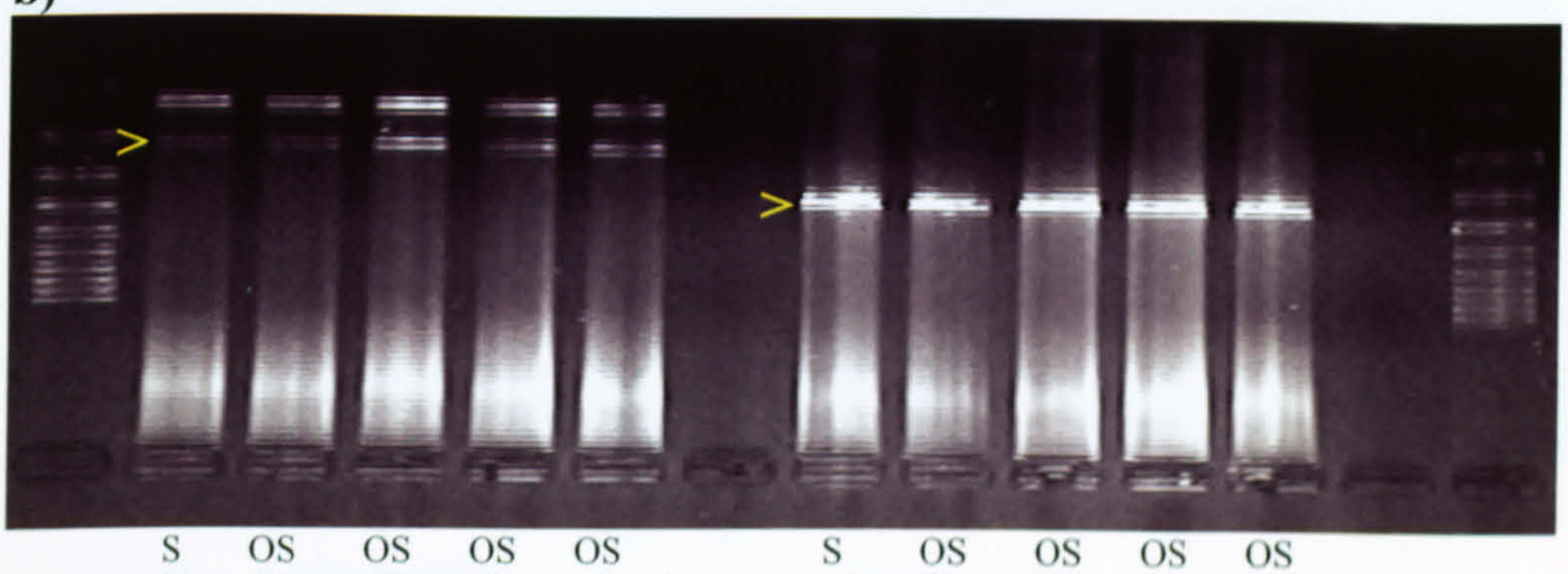


c)

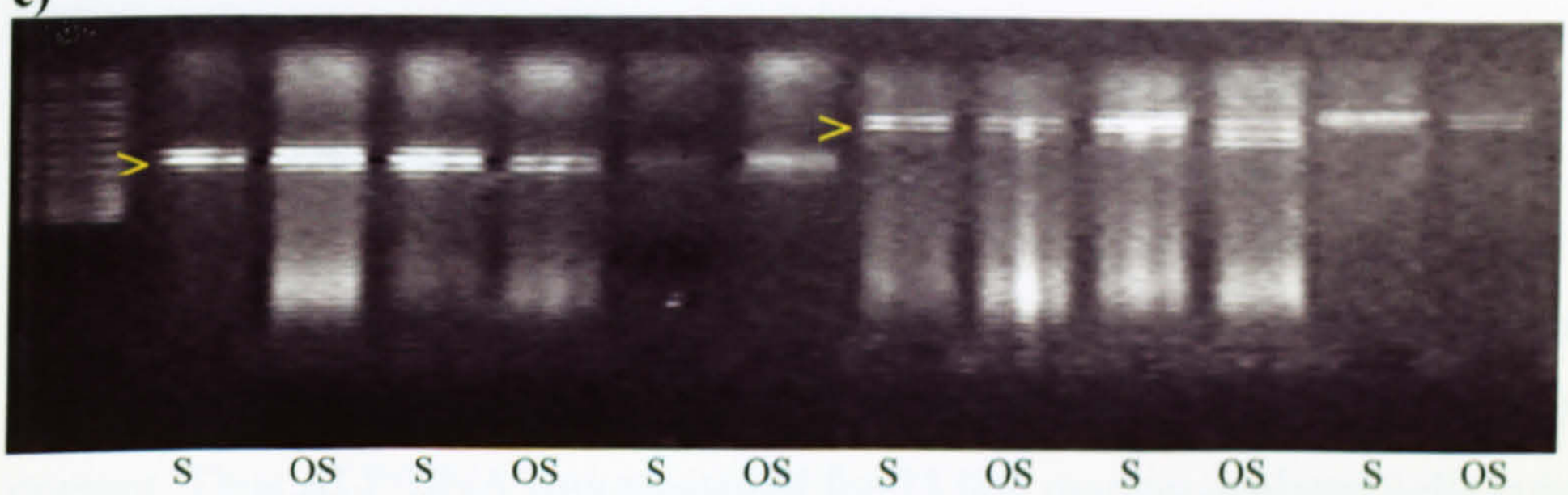
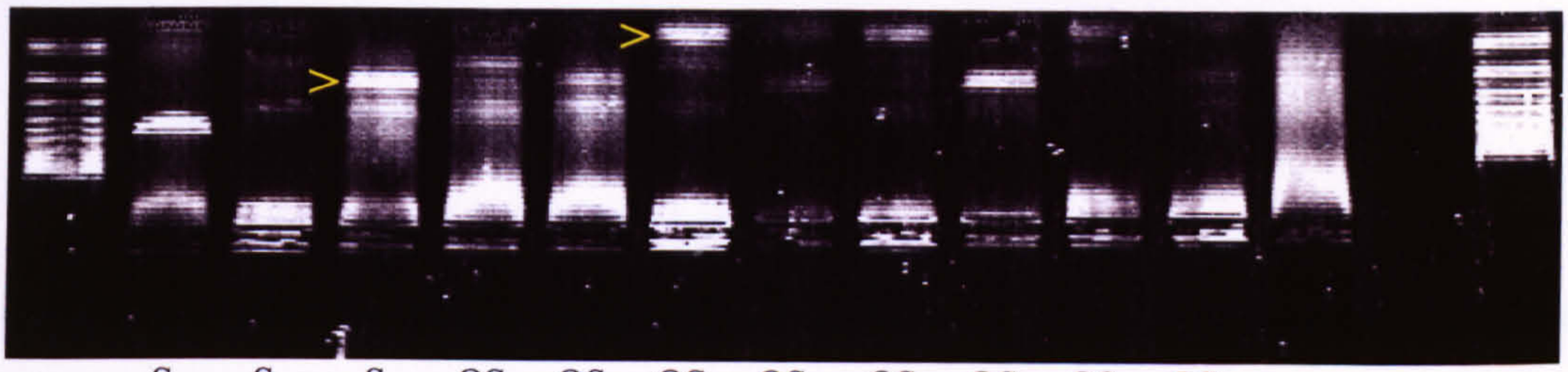


Figure 1. 11: Pictures of electrophoresis gels for the expression of Osteopontin and Osteocalcin by bone marrow isolated cells over 7 days (d) in culture. For each gel, yellow arrows indicate gene bands of osteocalcin and osteopontin at the first time they are seen:

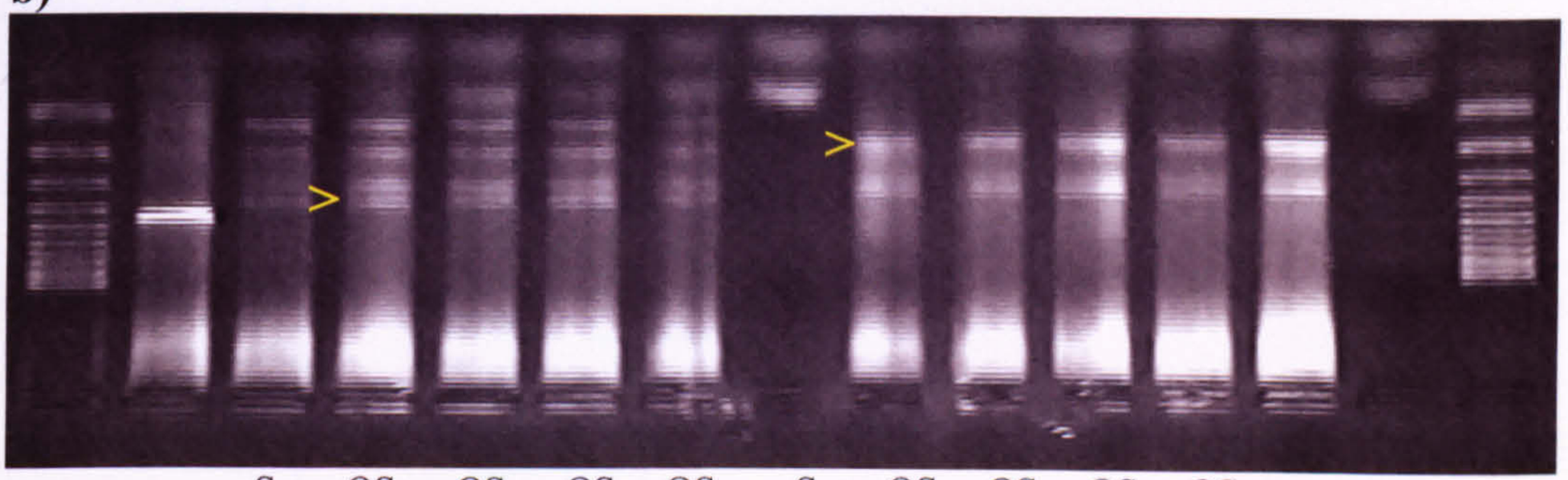
a) Patient 1: Gene expression with the addition of OS for osteopontin (OP) and osteocalcin (OC) compared with standard medium control (S) and Saos-2 positive control, GAPDH (G) control,

b) Patient 2: Gene expression with OS compared with standard conditions (S), for osteopontin (wells 3-7), osteocalcin (wells 9-13) and GAPDH (G, well 2).

a)



b)



2.3.3.4 The effect of osteogenic supplements on protein production

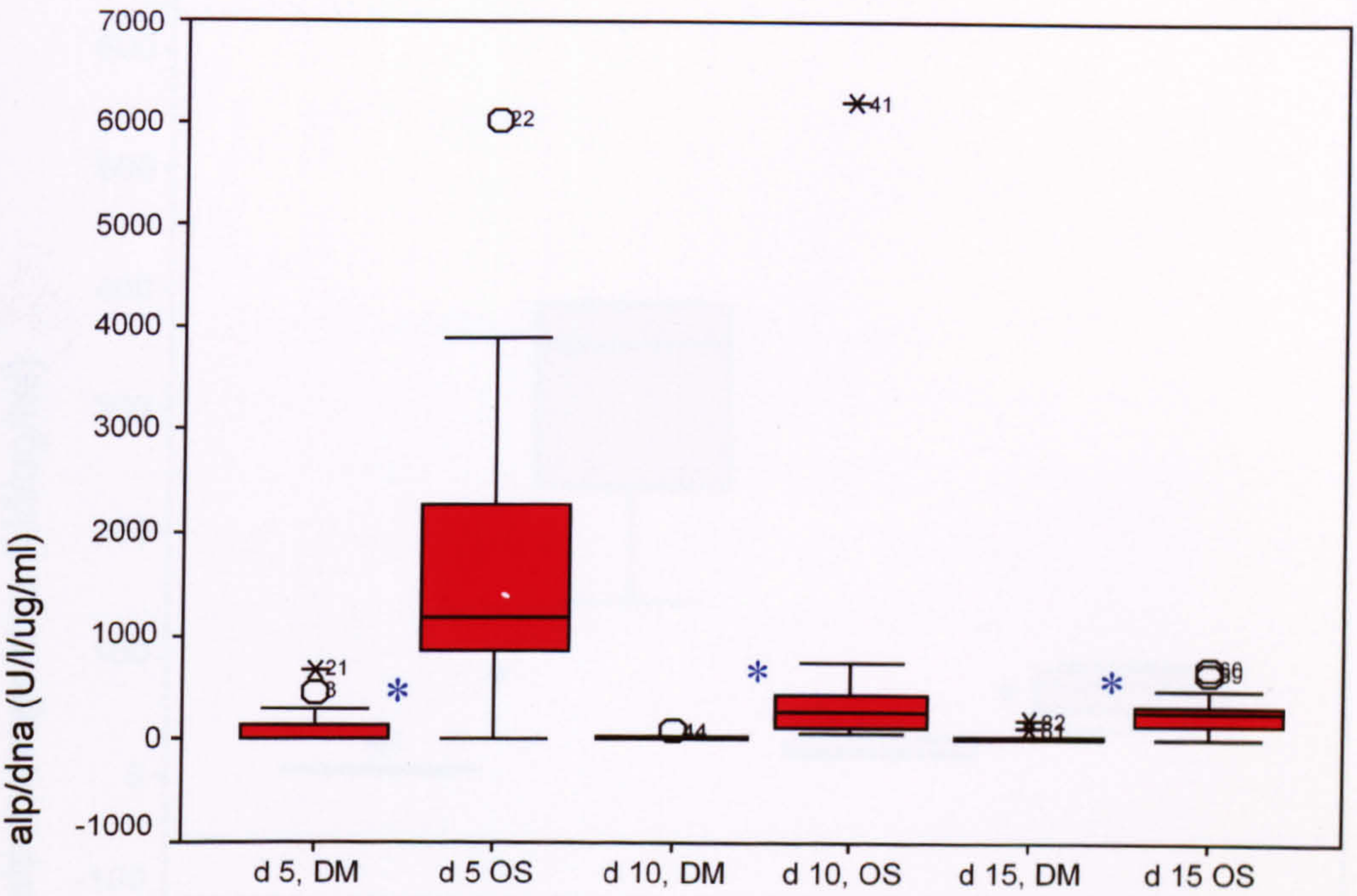
2.3.3.4.1 Alkaline phosphatase production over 15 days

As noted in the method section of this chapter, to allow statistical comparison between conditions, the production of ALP for each sample was divided by the total DNA content. Thus ALP/DNA was measured for 25,000 marrow-isolated cells cultured in monolayer over a 15-day culture period.

In standard culture conditions the cells were found to produce a minimal amount of ALP. However, following the addition of OS to the culture for 5, 10 & 15 days, the production of ALP significantly increased ($P<0.005$), (see figure 1.12). The peak increase in ALP was after 5 days in OS culture, following which the protein production reduced, although it remained significantly greater than the control throughout the culture period.

There were a number of outliners and extreme results, as seen on the box plot in figure 1.12. In the majority of cases these were found to be close to the 95% confidence bands. However for each of the ALP results after both 5 and 10 days in OS culture, the outliner/extreme result was extremely high. As, in each case, only one result fell outside the trend these are likely to be freak results.

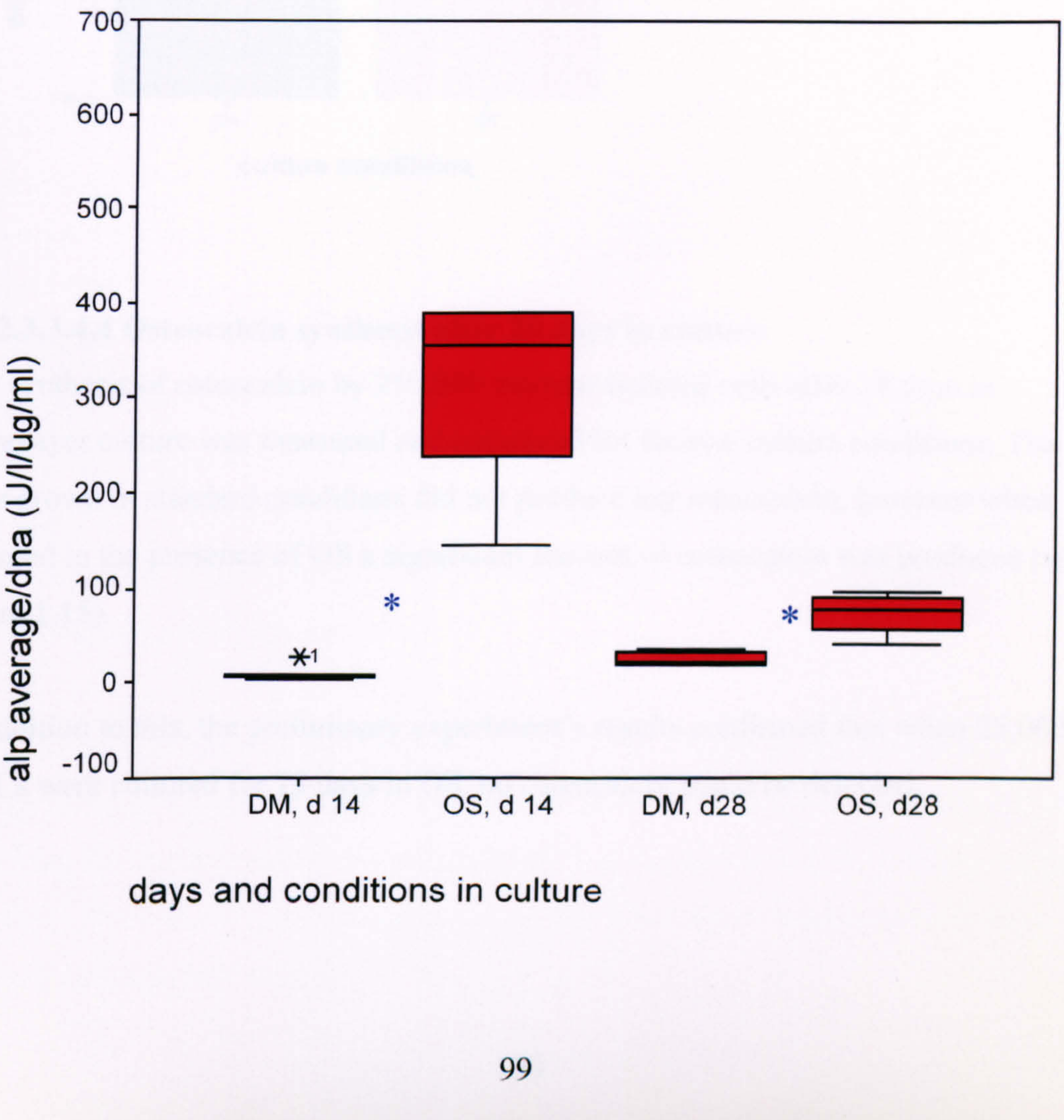
Figure 1. 12: A box plot comparing ALP expression (U/l)/DNA content (μ/ml) for marrow isolated cells cultured in OS with control population (DM) over 15 days, * $P<0.005$, (^{o}outliners, *extremes).



2.3.3.4.2 Alkaline phosphatase production over 28 days

The addition of OS to the culture was also found to significantly increase the production of ALP/DNA ($P<0.05$), when a larger cell seeding density (250,000 cells) was used and the marrow cells were cultured over a longer culture period (28 days) in monolayer, (see figure 1.13). The production of ALP remained minimal over the culture period in standard conditions, consistent with the previous experiment, confirming that the addition of OS was necessary for increased production of ALP. The increase in ALP was greatest 14 days through this culture period, although it remained significantly higher for cells in OS through out the culture. As seen in figure 1.13, there were no outliners or extreme results, suggesting that such results seen in figure 1.12 were freaks as discussed above.

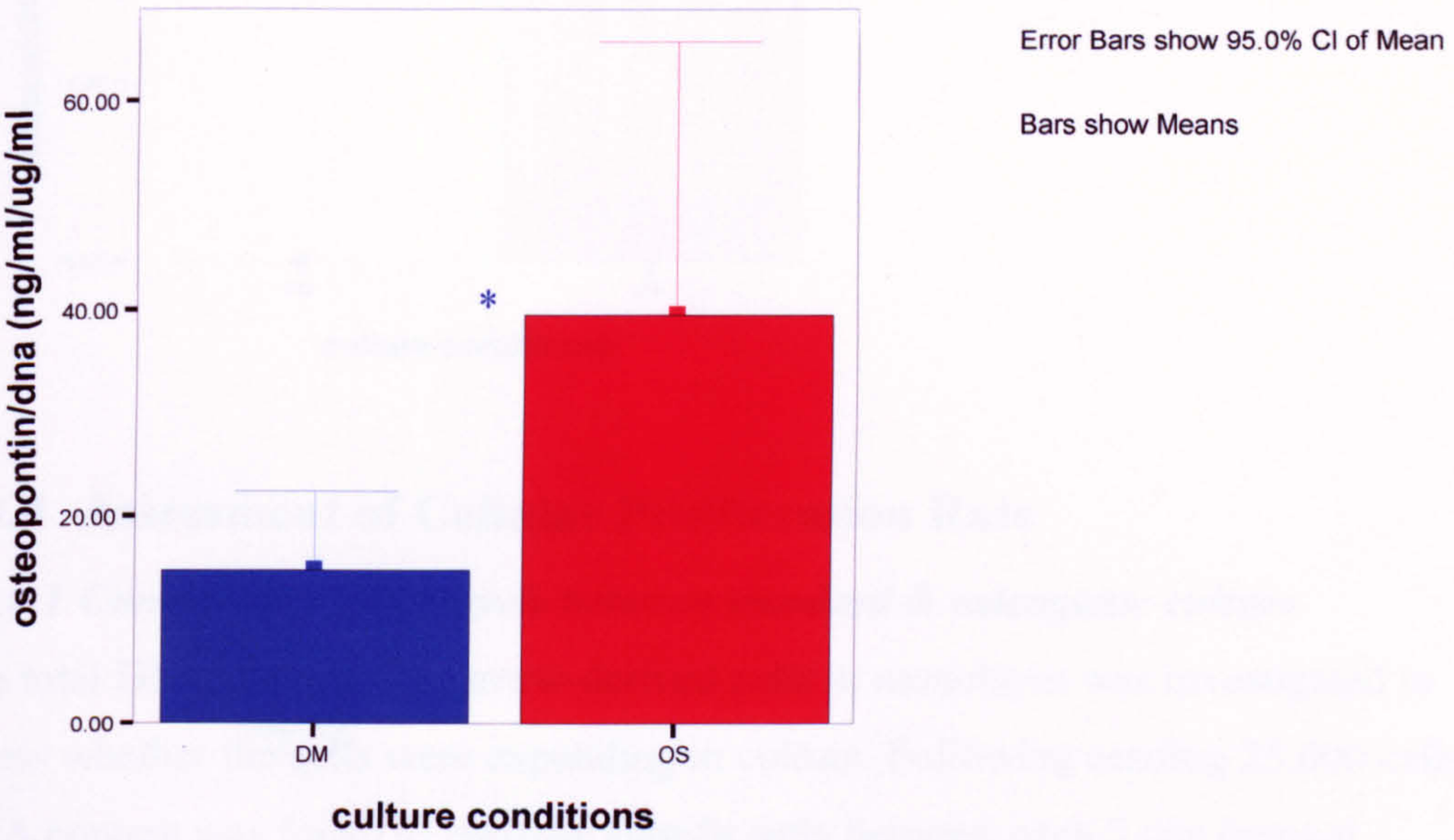
Figure 1. 13: Box plot of the effect of OS compared with standard conditions (DM) on ALP production by MSCs, * $P<0.05$.



2.3.3.4.3 Osteopontin production by marrow isolated cells in monolayer

When 250,000 marrow-isolated cells were cultured for 14 days with OS, the synthesis of osteopontin increased significantly compared with the control ($P<0.05$), (see figure 1.14).

Figure 1. 14: Bar chart comparing osteopontin (ng/ml)/DNA content (μ/ml) for marrow cells cultured in OS with control population after 14 days, * $P<0.05$.

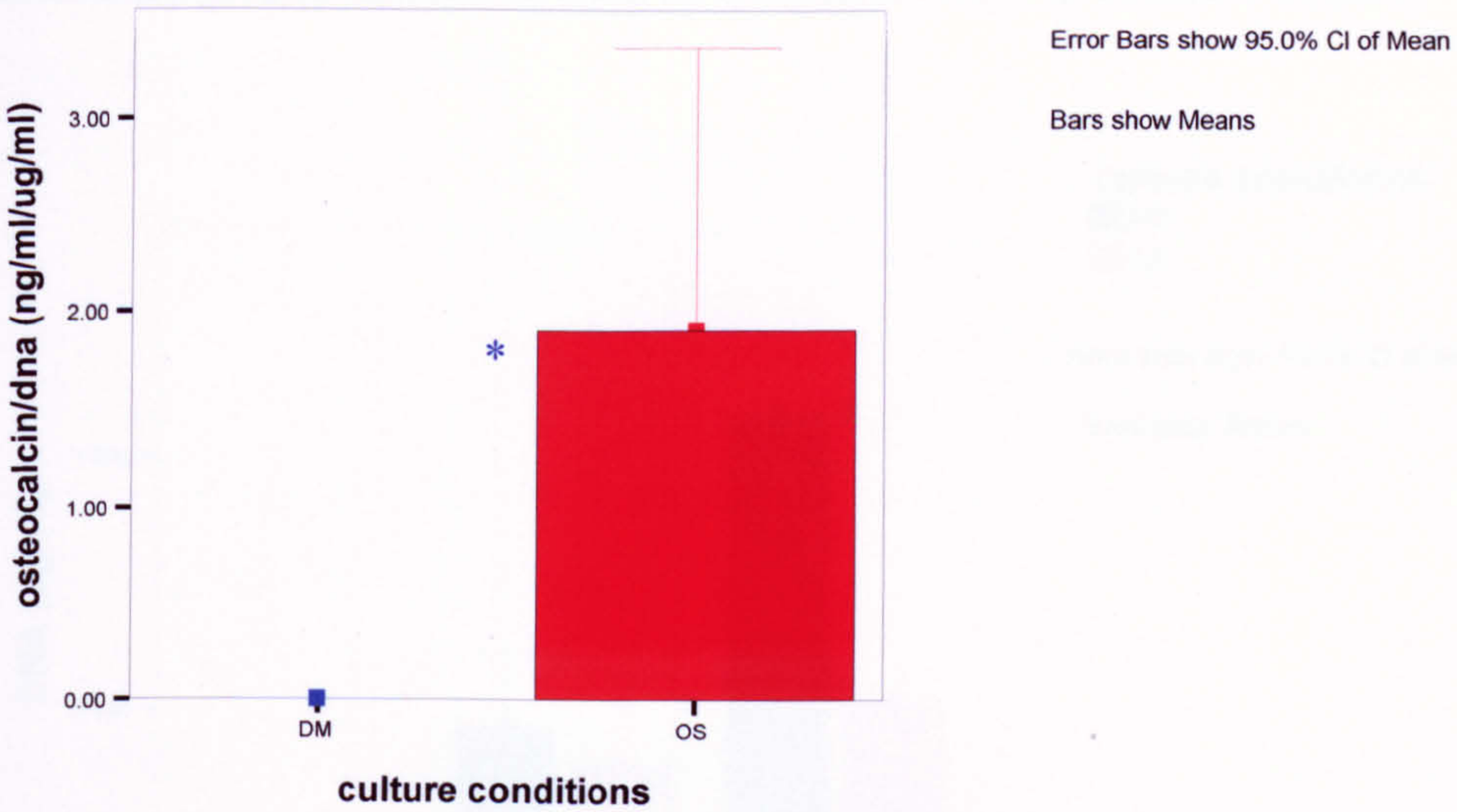


2.3.3.4.4 Osteocalcin synthesis after 28 days in culture

The synthesis of osteocalcin by 250,000 marrow-isolated cells after 28 days in monolayer culture was measured and compared for the two culture conditions. The cells grown in standard conditions did not produce any osteocalcin, however when cultured in the presence of OS a significant amount of osteocalcin was produced (see figure 1.15).

In addition to this, the preliminary experiment's results confirmed that when 25,000 MSCs were cultured for 15 days in OS, no osteocalcin could be detected.

Figure 1. 15: Bar chart showing the effect of OS on osteocalcin production after 28 days in cell culture, * P<0.05.



2.3.4 Assessment of Cellular Proliferation Rate

2.3.4.1 Comparative DNA levels between standard & osteogenic culture

The total DNA content of marrow-derived cells in monolayer was investigated to assess whether the cells were expanding in culture. Following seeding 25,000 cells, the DNA content was found to increase significantly between each 5 day interval measured for the cells cultured in standard conditions over 15 days (P<0.005), (see figure 1.16).

A similar relationship was found when the seeding density was increased to 250,000 cells, as the amount of DNA significantly increased between 14 and 28 days (P<0.05), (see figure 1.17). When an initial seeding density of 250,000 cells was used, after 14 days in standard culture, the amount of DNA was greater than for 25,000 cells after 15 days in culture, as would be expected.

The DNA results were significantly greater for cells cultured in standard medium compared with OS, after 15 days following the lower seeding density and after 28 days following the higher seeding density (P<0.05), (see figures 1.15 & 1.16). This suggests that DNA levels did not increase at the same rate when marrow-derived cells were cultured in osteogenic medium.

Figure 1. 16: Bar chart illustrating the increase in DNA content when 25,000 cells were cultured in standard medium (DM), between 5 and 10 days and between 10 and 15 days, ^P<0.005. Also, the effect of OS compared with DM was related to a significantly greater amount of total DNA after 15 days in standard culture, * P<0.05.

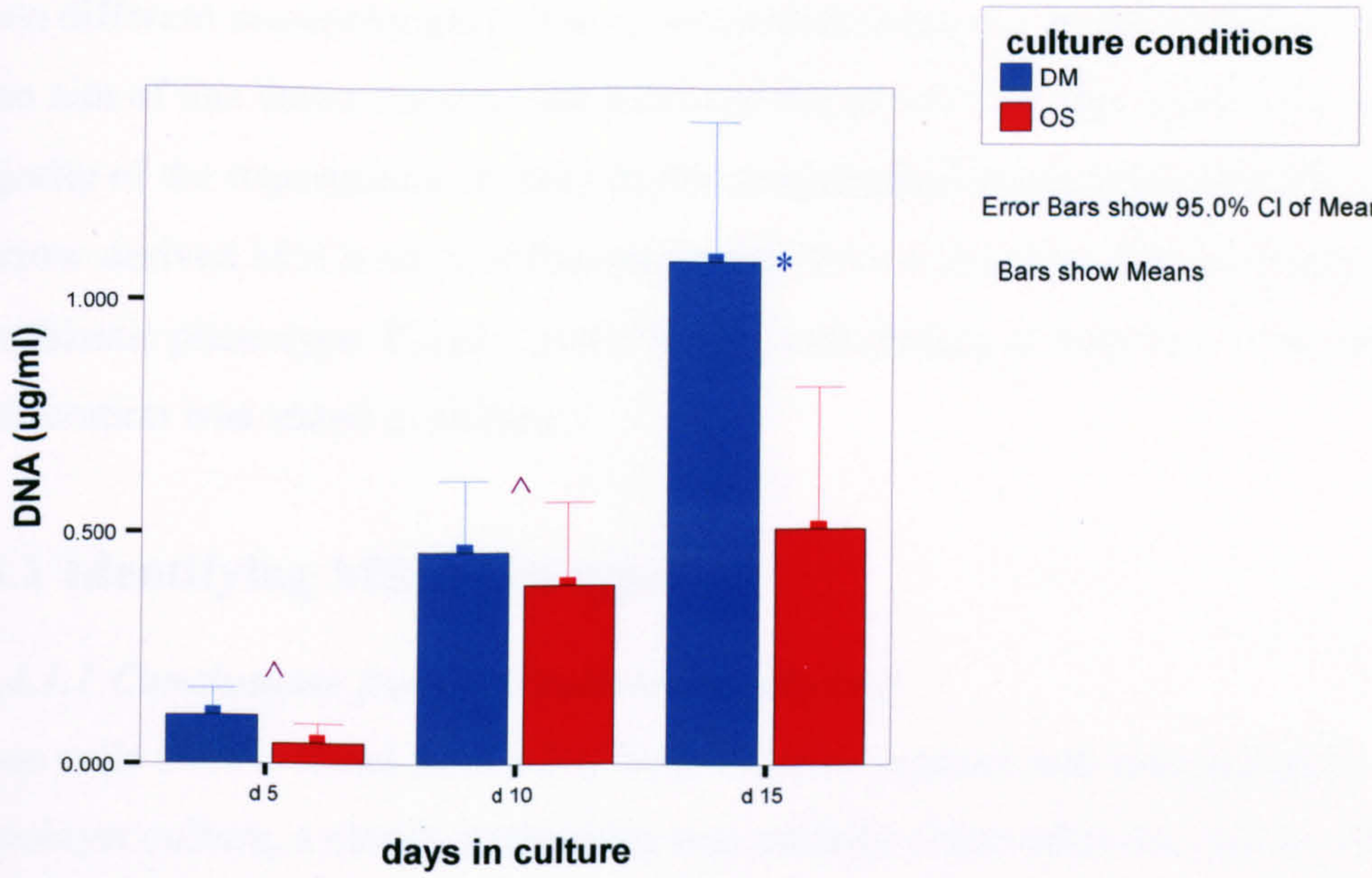
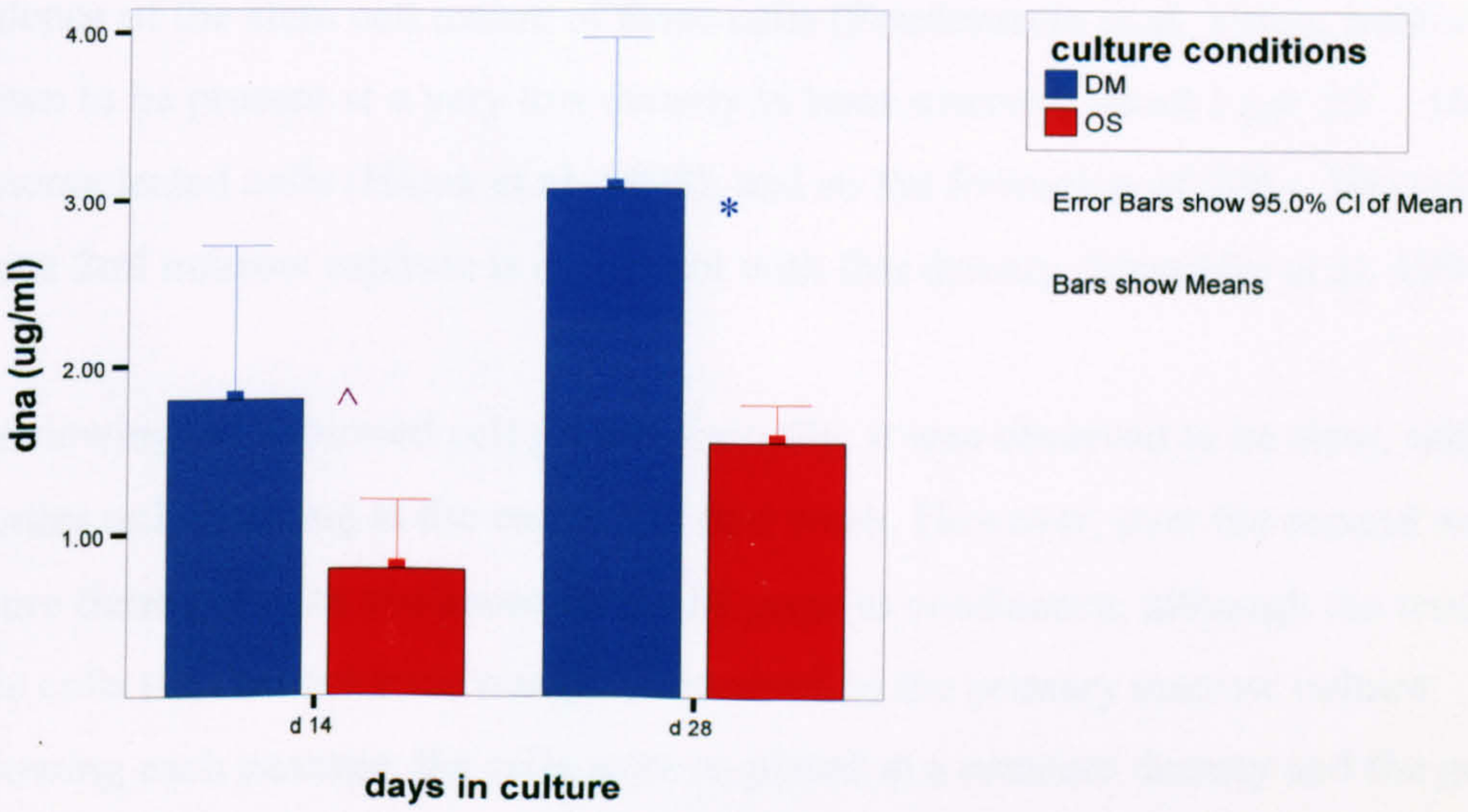


Figure 1. 17: Bar chart illustrating the increase in DNA between 14 and 28 days, on 250,000 cells seeded in standard medium (DM), ^P<0.005. Also showing the greater total amount of DNA after 28 days in DM culture compared with OS, * P<0.05.



2.4 DISCUSSION

In this chapter, it was hypothesised that MSCs could be isolated from human bone marrow aspirates and further identified as MSCs, due to their ability to differentiate down different mesenchymal cell lines when stimulated in culture. Furthermore, as the main aim of this thesis involves the potential use of MSCs in the repair of bone, the majority of the experiments in this chapter concentrated on showing that these marrow-derived MSCs could differentiate into cells expressing characteristics of the osteoblastic phenotype. Finally, as the MSCs' cell density is very low in marrow, cell proliferation was tested in culture.

2.4.1 Identifying MSCs cell type

2.4.1.1 Conclusions from cell culture morphology

When cells were isolated from a 2ml bone marrow aspirate and were plated in monolayer culture, a cloudy suspension was initially observed in the culture flasks, however this cleared following two medium changes. Thus this was likely to be due to the non-adherent haematological cells, the majority of which were erythrocytes.

The cells were observed to adhere and form 150-250 colonies per flask, with each colony containing up to 500 spindle-shaped cells. This suggests that each colony was the result of multiple divisions of a single cell, this behaviour was proposed as evidence of the stem cell nature of these cells (Friedenstein et al. 1966). MSCs are known to be present at a very low density in bone marrow, about 1 per 10^5 - 10^6 mononucleated cells (Hicok et al. 1998), and so the formation of 200 – 300 colonies from a 2ml marrow aspirate is consistent with this density (Muschler et al. 1997).

In reviewing the aspirated cell growth, initially, it was observed to be slow, with colonies only forming at the end of the first week. However, over the second week in culture these colonies coalesced and cells grew to confluence, although the tendency of these cells to form colonies was only observed in the primary marrow culture. Following each passage, the cells were re-plated at a constant density and the growth rate was found to increase after the first 2 passages, but then remained constant to passage 5. The cells were further sub-cultured over 10 passages with no obvious

change to their morphology. These observations are similar to those described by Friedenstein (Friedenstein et al. 1970) and as described with rat MSCs (Goshima et al. 1991b).

SEM confirmed that isolated bone marrow cells were fibroblastic-like in morphology, which is consistent with the expected morphology of MSCs, but as morphology is not specific to cell type further indicators of MSCs were used.

2.4.1.2 Use of Stro-1 in the identification of MSCs

All the cells that were isolated from each of the four patients' bone marrow aspirates were strongly positive for Stro-1 on immuno-florescence testing. This is evidence that the marrow-isolated cells possessed the MSC phenotype, as marrow stromal precursor cells are Stro-1 positive cells (Simmons & Torok-Storb 1991). Stro-1 has been used previously as a marker for MSCs (Gronthos et al. 1994) and although it is not totally specific as it is also present on leucocytes, it does not bind to haematopoietic progenitor cells, which may also be present.

As Stro-1 is a marker of uncommitted precursor cells, it would be expected that the intensity of the Stro-1 identification of the cells would decrease, when the MSCs were stimulated to differentiate. When MSCs were cultured with OS the shape of the cells was noted to change, becoming consistent with osteoblasts. However, the cells continued to express the cell surface antigen identified by Stro-1 although, on subjective assessment, the fluorescence intensity decreased, which may reflect differentiation of the marrow cells into committed osteoblast progenitors, thereby lowering Stro-1.

The positive control, MG-63 osteosarcoma cell line, was less positive than the marrow-isolated cells and produced results similar to the OS stimulated cells. This is further evidence that the marrow-isolated cells were MSCs and that OS stimulated differentiation with relative loss of Stro-1. The negative control Saos-2 was not found to express Stro-1. These control results were also as predicted (Stewart et al. 1999), thus confirming the accuracy of the experiment.

In summary, the marrow-isolated cells used in this thesis were found to be positive for Stro-1, a marker of MSCs.

2.4.2 Differentiation down mesenchymal cell lines – Histological stains

As it has been suggested that Stro-1 is not a totally specific marker for MSCs and, as discussed in Chapter 1: Markers of MSCs section, marker specific to MSCs have been difficult to identify, the characterisation of the marrow-isolated cells was further investigated by differentiation of the cells down mesenchymal cells lines.

2.4.2.1 Osteoblasts identified by Alkaline Phosphatase stain

Alkaline phosphatase (ALP) is known to be expressed by maturing osteoblasts. Furthermore, Naphtol-AS-B1 sodium phosphate stain and fast red violet has been used previously to stain both human osteoblasts (Matsuyama et al. 1990) and differentiated MSCs in culture for the presence of ALP (Ohgushi et al. 1996b).

The majority of marrow-isolated cells that had been cultured with OS for 7 days stained positively for ALP within the cytoplasm and cell membrane. The staining correlated with the change in shape as the cells became less spindle-like, which suggested a change in phenotype, as characterised by the expression of ALP. It also seemed that the majority of the cells were stimulated to produce ALP, rather than a small number of the cells expressing large amounts of ALP; the stain differentiated between these two circumstances, both of which would lead to an increase in ALP protein levels, as detected by biochemical assay.

It would be expected, following the stem cell theory as discussed in Chapter 1, that some cells would retain the stem cell pool, remaining immature. It was found that the occasional fibroblastic cells did not stain for ALP despite 14 days culture in OS. This suggests that, even in osteogenic conditions, not all the marrow-isolated cells were stimulated to differentiate and that cells divided to maintain a progenitor population, thereby continuing the overall multipotent nature of the culture.

In comparison, few of the marrow-isolated cells, cultured for the same time period in standard medium, stained slightly positively for ALP and the cells remained

fibroblastic in shape. This suggests that, without the influence of OS, the marrow-isolated cells did not produce ALP. The Saos-2 osteosarcoma cell line was cultured in the same standard conditions as a positive control and, as expected, these cells stained for ALP to the greatest extent.

From these results it can be concluded that the bone marrow-derived cells produce ALP, a marker of the osteoblastic phenotype, in response to culture with OS. However, the use of a histological stain allows only observational results.

2.4.2.2 Mineralisation identified by Von Kossa stain

As the primary function of osteoblasts is to produce bone, this function can be used to define the cell type. Mineralisation of tissue samples from *in vivo* experiments has been assessed histologically (Haynesworth et al. 1992b), and electron microscopy techniques have also been used on *ex-vivo* implants to assess mineralisation of the extracellular matrix produced by cells (Bab et al. 1988b) in order to characterize the osteoblastic phenotype.

Although mineral deposits have been reported associated with osteoblasts *in vitro* (Binderman et al. 1974), assessing mineralisation of extracellular matrix in tissue culture is more difficult. This is the result of less extracellular matrix forming in monolayer culture than within tissue. However, when 3-d bone nodules have been stained for the presence of extracellular calcification using the von Kossa technique, the stain has been shown to correlate with the detection of collagen type I and osteonectin by immunochemistry (Bellows et al. 1986). Consequently, von Kossa staining has also been used to identify the formation of mineralised nodules *in vitro* by human bone marrow-derived cells (Cheng et al. 1994).

In this study, cells were seeded at a high density and a long culture period was used to encourage stratification of the cells. After 21 days in OS, von Kossa staining indicated calcification within the culture, which was observed to have increased by 28 days. The extracellular matrix stained to a lesser extent than for the Saos-2 control, although mineralisation was also limited in this culture. As discussed, it has been suggested that

osteoblasts require a 3-d structure in order to produce a mineralised matrix. Therefore, an explanation for the limited mineralisation may be that the cells was cultured on a 2-d surface. There was no positive staining for mineralisation of the MSCs cultured in standard medium, as expected.

In conclusion, some mineralisation did result from the culture of marrow cells in OS, thereby suggesting matrix production by osteoblasts, and the presence of osteoblast precursors in cultured marrow-isolated cells.

2.4.2.3 Adipocytes identified by Oil red O

In addition to osteoblastic differentiation, the marrow-isolated cells were also tested for adipocytic differentiation, by culturing the cells in medium containing adipocytic supplements (Pittenger et al. 1999). These cells were stained using oil red O for intracellular lipid, which forms large droplets in adipocytes. Although, some lipid was present within the marrow-isolated cells cultured after 7 days in standard medium, this is often found in cells in culture. The size of cells that had been stimulated to differentiate down the adipocytic cell line were large due to increased cytoplasm, consistent with adipocytes and contrasted with the spindle shape of the marrow cells that persisted in standard culture. Although after 14 days in adipocytic medium, the majority of cells were observed to contain multiple large lipid droplets, no change was detected in cells in standard culture.

In conclusion, cells isolated from human bone marrow could be stimulated to differentiate into adipocytes, as defined by a large frequency of intracellular lipid droplets.

2.4.2.4 Chondrocytes identified by Alcian blue & Sirius red

Alcian blue/sirius red were used to stain collagen matrix produced by cells in culture, as it detects the lattice arrangement of collagen fibres, which polarises under polarised light, as organised in tissue.

When the marrow-isolated cells were cultured in standard conditions for 14 days, no collagen was detected and the cells maintained their spindle-like shape. Similarly,

although cells cultured in osteoblastic conditions produced no collagen matrix, they were less fibroblastic in morphology after 14 days. However, when marrow cells were cultured with supplements known to stimulate chondrogenic differentiation (Johnstone et al. 1998), the cells formed clumps and a polarising collagen matrix was observed surrounding every clump. Bovine chondrocytes were cultured as a positive control. These were not found to produce collagen, which could be due to the culture conditions, whereby the cells were grown in monolayer.

It is noted that alcian blue/sirius red is not specific to collagen type II, which is produced by chondrocytes and as osteoblasts produce collagen type I, this stain will not differentiate between these two cell types. However, marrow cells cultured in osteoblastic conditions did not stain for collagen lattice, but in chondrocytic conditions a lattice was observed. In order for these cells to produce an organised collagen lattice, the cells need to grow in a 3-d arrangement. Therefore, it seems that the chondrogenic supplements stimulated the cells to form clumps and produce an organised collagen lattice, suggestive of chondrocytic differentiation. Further work to clarify this would require identifying specifically collagen type I or II on cells cultured in either osteogenic or chondrocytic medium.

These results indicate that marrow-isolated cells, when cultured with chondrogenic supplements, were stimulated to produce a collagen matrix, consistent with chondrocytes. Taken together, my results suggest that marrow-isolated cells can be differentiated down the chondrocytic, adipocytic and osteoblastic cell lines, providing evidence of their multipotent nature indicating that they are MSCs.

2.4.3 Further investigation of the differentiation of MSCs into osteoblastic cells

2.4.3.1 Conclusions from cell culture morphology

The addition of OS to isolated bone marrow cells in culture resulted in an observed change in morphology under light microscopy. The cells changed from long spindle cells, recognised to be MSCs, to shorter, brick-shaped cells after 7 days in these culture conditions. Osteoblastic cells, either a primary cell line extracted from trabecular bone or an osteosarcoma cell line, are square in shape when observed under light

microscopy (Aubin & Liu 1996; Liu et al. 1997; Lomri et al. 1988; Malaval et al. 1999). The observed change in morphology is therefore consistent with the bone marrow-derived cells becoming osteoblastic.

It has been noted that fibroblasts in cell culture can lose their spindle shape resulting in flattened cells that may look similar to those seen in these cultures with OS. However, if the observed change in morphology was due to this, the cells in standard medium would also be expected to change. Thus, since in this experiment cells in standard medium maintained their spindle shape, it is suggested that the change in shape was due to differentiation.

2.4.3.2 Conclusions from SEM results

Similar changes in morphology were observed under SEM. After 24 hours in culture on a therminox surface, the cells cultured in OS were cuboidal in shape compared to the control culture cells, which remained spindle-shaped. The comparative difference in shape increased after 7 days. These findings were consistent with those observed under light microscopy.

In summary, only the isolated bone marrow cells stimulated by OS changed in shape, as observed under light and scanning electron microscopy. Although morphology is non-specific observational information, this change is consistent with the cells differentiating into osteoblasts when cultured in OS conditions. Hence, it can be suggested that the isolated bone marrow cells contain osteoblast precursor cells or MSCs that can be stimulated to differentiate down the osteoblastic cell line. However, osteoblastic differentiation was further defined in the following studies.

2.4.4 Use of Messenger RNA expression to detect osteoblastic differentiation

2.4.4.1 GAPDH

GAPDH is a housekeeping gene that is expressed by all human cells. Therefore, it was used as a positive control and all the cell samples studied using RT-PCR were found to

express GAPDH. This confirmed that mRNA was extracted from each sample and that the method used detected this correctly.

2.4.4.2 Cbfa-1

Cbfa-1 is an early marker of osteoblastic differentiation, as outlined in the Introduction to this chapter, as it acts as a transcription factor for the expression of osteocalcin and is essential for the ossification of bone (Ducy 2000). Therefore, it would be expected that the Cbfa-1 gene would be transcribed in osteoblast progenitor cells and this would persist in differentiating cells, then reduce marked by the cells starting to produce osteocalcin.

All the marrow-derived samples that had been cultured without OS were found to express Cbfa-1, which suggests that MSCs express Cbfa-1 when cultured in standard conditions. A further explanation of these results would be the presence of osteoblast progenitor cells as part of a heterogeneous group of progenitor and stem cells in this population.

Cbfa-1 expression increased on the addition of OS in every sample of cells with the expression peaking after 3 days in OS and decreasing to a minimal level after 7 days in culture. This suggests that cell signalling stimulated the cells to express maximal amounts of Cbfa-1 after about 3 days in OS, which indicates that these cells were differentiating into osteoblast progenitor cells. The amount of Cbfa-1 detected then decreased by day 7, as levels of the transcription factor reduce with osteoblastic maturation, this suggests that the cells were being stimulated to differentiate further down the lineage. This proposal was supported by my results that showed that osteosarcoma cells, which were used as a positive control for osteoblasts, expressed lower levels of Cbfa-1, confirming that differentiated osteoblastic cells express this gene to a lesser extent.

2.4.4.3 Osteopontin

The effect of OS on the expression of osteopontin was assessed over 7 days in culture using RT-PCR.

The cells from one patient's marrow cultured in standard conditions expressed osteopontin. However the other patient's cells did not express osteopontin until cultured with OS. As osteopontin is expressed by proliferating cells (Lian & Stein 1992), if the first sample of MSCs was dividing more rapidly than the second sample, this could account for the expression of osteopontin. A further explanation could be that, in addition to MSCs, this cell population included osteoblast progenitor cells which express osteopontin (Roach 1994), as suggested above. To clarify this, marrow-derived cells from a larger sample of patients need to be studied.

Following culture with OS, osteopontin mRNA was detected at each time point measured. Thus, when MSCs are cultured with OS they change into cells that express osteopontin over a 7-day period, which is consistent with differentiation down the osteoblastic lineage. It has been suggested that the expression of osteopontin by osteoblasts is biphasic, with the first peak relating to proliferation and the second to osteoblastic differentiation (Aubin et al. 1995). Therefore, to investigate whether this pattern is seen as MSCs differentiate into osteoblasts, further studies of more patient samples over a longer culture period would be needed.

2.4.4.4 Osteocalcin

Marrow-isolated cells in standard medium were not found to express osteocalcin, signifying undifferentiated cells as expected in these cultures. This is consistent with the findings that the osteocalcin gene was not found to be expressed by bone marrow cells (Falla et al. 1993) or osteoblast progenitor cells (Aubin 1998), in culture. Osteocalcin mRNA from patient 1 was expressed after 3 days in OS culture and after 1 day for patient 2's sample. Both samples continued to express osteocalcin for the remainder of the culture in OS. As gene expression of osteocalcin has been found to correlate with cells that form mineralised tissue (Mizuno & Kuboki 2001), these results indicate that OS increased osteoblast gene expression.

In summary, Cbfa-1, osteopontin & osteocalcin can be used as markers of increasing osteoblastic differentiation. Furthermore, the sequential expression of these genes, following the addition of OS to marrow-derived cells in culture, supports the hypothesis that OS stimulates MSCs to differentiate into osteoblasts.

The main criticism of these RT-PCR results is that, although they confirm that the population of cells cultured in OS express mRNA known to be expressed by osteoblasts, the results do not quantify this at cellular level. Therefore, the increase could be due to a small number of cells synthesising mRNA, being stimulated by OS to produce a larger amount. These cells could be osteoblast progenitor cells present in the marrow at aspiration. However, the histological stains provide evidence that, cells in standard medium did not produce ALP, in contrast the majority of cells in OS did then produce this osteoblastic protein. Taking these results together suggests that the majority of marrow-derived cells differentiate down the osteoblastic lineage, when cultured in osteogenic conditions.

2.4.5 Protein production to determine differentiation into osteoblasts

2.4.5.1 Alkaline phosphatase (ALP)

The amount of ALP protein was measured quantitatively to assess osteoblastic activity in culture. This method has been used to assess osteoblastic maturation of clonal cell lines, MC3T3-E1 (Sudo et al. 1983), differentiation of rat MSCs in culture (Owen et al. 1991) and differentiation of human MSCs down the osteoblastic lineage *in vitro*, by many investigators (Cheng et al. 1994; Jaiswal et al. 1997; Maniatopoulos et al. 1988).

Although, using histological staining, ALP was shown to be present in the cytoplasm of the majority of MSCs cultured in OS after 7 days, the increase in ALP protein levels by cells cultured in OS over time could be explained by an increase in the total number of cells, rather than an increase in the expression of ALP by individual cells. Therefore, as outlined in the method, to standardise the results for the number of cells, the total DNA content of each sample was measured. The ALP expression was divided by the total DNA content allowing comparisons to be drawn between samples in two conditions. The same was true for the other proteins measured in my studies.

The results of my study showed that minimal amounts of ALP were produced when either 25,000 marrow-isolated cells were cultured over 15 days or when 250,000 cells were cultured over 28 days in standard medium. Thus, in control conditions, some cells produced low levels of ALP, but production did not increase over time.

ALP has been used previously to define the stages of differentiation down the osteoblastic lineage, as it has been found to be expressed early in the postulated sequence of osteoblastic development and has therefore been suggested as a marker of osteoblast progenitor cells (Turksen & Aubin 1991). The results of my study are consistent with the standard culture containing both MSCs, which do not produce ALP, and few osteoblast progenitor cells that produce low levels of ALP. As production of ALP remained constant in standard conditions, the cells were not differentiating into osteoblasts and the proportion of osteoblast progenitor cells thus remained constant. This observation, that ALP positive cells have osteoblastic potential, has lead others to isolate such cells from marrow cells for studies into osteoblast progenitor cells (Bruder & Caplan 1990; Walsh et al. 2000).

In this chapter, the addition of OS to 25,000 marrow-isolated cells significantly increased the expression of ALP measured after 5 days as compared with the control ($P<0.005$). This significant difference was maintained over the 15-day culture period ($P<0.005$). The expression of ALP peaked at day 5, which is consistent with reports in scientific literature that have shown ALP protein expression is an early marker of osteoblastic differentiation (Aubin et al. 1995) and, more specifically, MSCs in osteogenic culture have been shown to produce significantly higher levels of ALP after 5 days (Zvaifler et al. 2000).

When a larger number of MSCs (250,000) were cultured in the presence or absence of OS the same response was confirmed as, after 14 days (first time point measured) in osteogenic conditions, cells expressed a significantly higher level of ALP ($P<0.05$). The significant increase in ALP production was maintained until the end of a 28-day period ($P<0.05$), although the amount of ALP produced by the cells in OS decreased. The expression of ALP has been shown to occur before mineralisation of bone matrix and hence its presence has been implicated in initiating this process (Hui et al. 1997). It would therefore be expected that, following the peak of ALP production (within the first week in OS culture in these studies), the cells would be primed for mineralisation.

ALP is also used as a clinical marker of bone turnover and the amount of protein can be measured in serum (Eyre 1997). However, ALP protein production is not specific to osteoblastic cells, as it is also expressed by fibroblasts and chondrocytes (Johnstone et

al. 1998). As a result of this, production of other proteins produced by osteoblasts was also measured in my study.

2.4.5.2 Osteopontin

Osteopontin is produced by osteoblasts during embryonic intramembraneous and endochondral ossification (Dodds et al. 1995). It has also been shown to be produced by osteoblasts ahead of the mineralisation front, where it forms part of the extracellular matrix prior to bone formation (Roach 1994). Therefore, it is a useful marker of osteoblastic differentiation and function and as a result of this, production of osteopontin protein was measured in my study.

After 14 days in culture, MSCs that had been exposed to OS expressed significantly more osteopontin than the control ($P<0.05$). In view of the peak in ALP production before this point, the osteopontin detected is likely to coincide with osteoblastic matrix production prior to mineralisation. Serial osteopontin measurements over a longer period of time would be needed to confirm whether MSCs differentiating into osteoblasts also produce osteopontin in a biphasic pattern as has been suggested for osteoblast progenitor cells (Aubin et al. 1995), as suggested for the RT-PCR results.

Although significantly larger amounts of osteopontin were measured after the cells had been cultured with OS, un-stimulated MSCs also produce low levels of the protein after 14 days, which infers that the mRNA result (see above) was not an anomaly, but rather that MSCs produce low levels of osteopontin or that the cell population contained a few osteoblast progenitor cells. To investigate this further, also the production over time of osteopontin by MSCs in standard conditions needs to be studied.

Despite osteopontin being produced by osteoblasts, it is non-specific to this cell type as it is also produced by chondrocytes (Dodds et al. 1995; Liu et al. 1994). However, these osteopontin results provide further evidence that isolated bone marrow cells can been stimulated to differentiate down the osteoblastic lineage by OS.

2.4.5.3 Osteocalcin

Osteocalcin is extracellular protein that is produced by mature osteoblasts and is uniquely associated with mineralised tissue (Aubin 1998; Malone et al. 1982). Consequently it is a more specific marker of osteoblasts than other protein assays used in this study. For example, it is not produced by chondrocytes (Nakase et al. 1994), improving its validity as marker of osteoblastic differentiation of MSCs.

Marrow-isolated cells cultured in standard conditions did not produce osteocalcin, signifying the lack of osteoblasts in this culture. This contrasts with the low levels of ALP and osteopontin produced by the cells in control conditions, which may be explained by the presence of a small number of osteoblast progenitor cells that express ALP and osteopontin, in the bone marrow-isolated cells in standard conditions. However, osteocalcin is not produced by osteoblast progenitor cells, but by differentiated osteoblasts.

Marrow-isolated cells cultured for 28 days in OS were found to express significantly higher levels of osteocalcin than cells in standard conditions ($P<0.05$), although the actual levels of osteocalcin were low. Therefore for MSCs to synthesise osteocalcin in culture OS was necessary.

The production of osteocalcin by rat MSCs commenced after 21 days in OS (Peter et al. 1998). As no osteocalcin was detected when 25,000 MSCs were cultured for 15 days in OS, it is likely that the low protein levels present when 250,000 cells were seeded was due to the short culture period. However, further research is necessary to clarify this.

The detection of proteins produced by cells is a more reliable marker of cell phenotype than mRNA expression. Additionally the use of protein assays allows protein levels to be easily quantifiable permitting comparison between groups.

In summary, marrow-isolated cells cultured in OS produced significantly higher levels of ALP, osteopontin and osteocalcin proteins than similar cells cultured in standard conditions, which confirms that cells isolated from bone marrow were stimulated to

differentiate into osteoblasts and that the isolated marrow cells contained osteoblast progenitor cells or MSCs.

In conclusion, the results of my studies in this chapter indicate that marrow-isolated cells could be differentiated down the mesenchymal cell lineages of osteoblasts, adipocytes and chondrocytes, which, in turn, therefore indicate that MSCs were initially isolated from human bone marrow. This concurs with the Stro-1 identification of the isolated marrow cells.

2.4.6 Cellular proliferation rate

2.4.6.1 DNA content

The total DNA content of marrow-isolated cells was compared over time in culture to assess whether these cells could be expanded in culture. Although the assay did not determine between alive and dead DNA, as dead cells do not adhere in culture the majority would have been removed with medium changes. Thus, DNA levels were equated to cell number and an increase in total DNA was used to assess cell growth.

When 25,000 MSCs were cultured for 15 days, the amount of DNA measured for each condition increased significantly between each 5-day interval ($P<0.005$). The same increase in DNA was noted between 14 and 28 days, when 250,000 cells were initially cultured ($P<0.05$). These results confirm that the cells were increasing in DNA content, hence expanding in number in culture.

The amount of DNA for the cells cultured in standard medium was greater than for OS after 15 days (25,000 cells seeded) and 28 days (250,000 cells) in culture ($P<0.05$). This suggests that the proliferation rate of marrow-derived cells is reduced when cultured in OS. Cellular proliferation has been found to reduce when cells differentiate (Stein et al. 1990) and my findings are consistent with this inverse relationship between proliferation and differentiation. Therefore these results, assessing cell proliferation, further indicate that cells grown in OS were differentiating.

CHAPTER 3

Tissue Engineering of Bone using Mesenchymal Stem Cells on a Hydroxyapatite Scaffold

3.1 INTRODUCTION

3.1.1 Background to chapter

As discussed in Chapter 1, bone loss, following tumour excision, fracture, or the need to fill bone defects during revision arthoplasty surgery or joint fusions, requires the use of bone grafts. However, the key source of grafts, autologous bone graft, has its own associated problems of donor site morbidity and limited amount available per patient.

Despite these problems autograft is currently considered to be the 'gold-standard', because of its osteoinductive and autologous nature. Allograft on the other hand, although possessing the structural properties of bone, has a greatly reduced osteoinductive nature as a result of processing, which removes progenitor and blood cells and, depending on the degree of cleaning used, can destroy growth factors. This processing is necessary to prevent immune rejection of the graft and reduces the risk of transmission of pathogens (Goldberg & Stevenson 1987).

As a consequence of these limitations, much research has been carried out into synthetic bone graft substitutes, but with limited success (Shah 2000), (see Chapter 1). This chapter investigates whether a biological bone graft substitute can be formed *in vitro*.

3.1.2 Tissue engineering

In vitro cell culture has traditionally used a flat environment to research the behaviour of cells. In these conditions the cells attach themselves to the culture surface in a single layer, becoming orientated in one direction. This is an artificial situation when compared with the 3-dimensional (3-d) environment *in vivo*, where cells are surrounded by other cells and extracellular matrix.

Tissue engineering is becoming a growing field of research, whereby cells are grown in situations that aim to recreate their natural environment more closely. The objective of this is to generate a 3-d cellular structure from cells *in vitro*, that could be implanted to reconstruct diseased or absent tissue that would not normally repair, thus restoring

function (Oreffo & Triffitt 1999). It was suggested in my Chapter 1 that skeletal tissue engineering requires cells on a scaffold with bioactive factors.

3.1.3 Cells

The advantages of using MSCs for the tissue engineering of bone are firstly, that MSCs can easily be harvested by bone marrow aspiration and secondly, their propensity for self-renewal as a source of osteoblastic cells, as shown in Chapter 2. Alternatively, mature differentiated cells could be used. However, osteoblasts cannot easily be harvested, as this requires the extraction of cells from trabecular bone (Bellows et al. 1986). Bone harvesting would also cause more donor site complications than marrow aspiration. Furthermore, differentiated cells have a determined half-life, without the ability for self-renewal, which in the case of MSCs, is maintained by a progenitor cell pool.

Previous studies have implanted whole marrow *in vivo* to increase bone healing (Connolly et al. 1991a; Delloye et al. 1998). However, marrow contains a mixture of cell types and an array of growth factors, which have the potential to influence cell behaviour. This influence can be reduced, since a large number of MSCs can be culture expanded from a small volume of marrow. Therefore, only a small amount of marrow is needed, reducing donor site morbidity and this would also allow autologous cells to be used in potential clinical applications. Therefore, marrow-isolated, culture expanded MSCs were used in this study.

3.1.4 Scaffold

In order to achieve robust 3-d cell growth, a scaffold substrate is required and the result of its interaction with the cells should generate tissue. Furthermore, the scaffold acts as a carrier for implantation of cells *in vivo*. The ideal properties of a scaffold for tissue engineering bone are:

- 3-d scaffold, to facilitate 3-d cell growth
- Biocompatibility, to minimize inflammatory response
- Reabsorbability, to allow its replacement
- Biodegradabily, without harmful effects

- Support of cell attachment, to allow carriage/transfer
- Osteoconductivity, to encourage integration between donor and host tissue
- Porosity, for capillaries ingrowth and vascularisation to prevent central necrosis
- Material should allow maximal, uniform loading and retention of cells
- Structural properties, similar to biological tissue
- Ease of handling by the surgeon, at a single procedure, in theatre
- Cheap (Bruder et al. 1994b)

To meet these requirements, the scaffold needs to be tailored to the cell type and tissue. Hence, for the tissue engineering of bone, the aim of the scaffold structure is to resemble that of cancellous bone. The scaffold needs to support the growth of osteoblasts and provide mechanical support when packed into a tissue defect, but also allow the diffusion of nutrients and the ingrowth of capillaries. As bone has a very high extracellular matrix to cell ratio, the scaffold used needs to replace this extracellular matrix, supporting the cells until they produce their own matrix.

3.1.5 Scaffold types

Below are a list and descriptions of different possible scaffold types.

3.1.5.1 Biological scaffolds

Allograft is known to become more osteogenic if impregnated with bone marrow prior to implantation (Burwell 1964), making this the most basic type of tissue engineering. However, this still requires the use of allograft, which has disadvantages associated with it (see Chapter 1). Below are other biological materials that have been considered for use as scaffolds.

3.1.5.1.1 Demineralised bone matrix

Demineralised bone matrix (DBM) (allograft stored in hydrochloric acid for 48 hours), has been found to be somewhat osteoinductive, as it induced the differentiation of MSCs into osteoblasts and, although not as beneficial as autologous bone graft, has been shown to heal segmental bone defects in rabbits (Tuli SM & Singh AD 1978) and

rats (Einhorn et al. 1984). DBM is known to contain a mixture of bioactive factors, such as BMPs, TGFβ and FGF.

In comparison, the mineral remaining in allograft seems to reduce the effect of these bioactive proteins in the graft. Thus, it seems that decalcification allows closer interaction between these proteins and host cells, increasing their osteoinductive effect. However, the use of DMB relies on the supply of allograft, which is limited.

One of the considerations for any bone graft substitute is the ease of handling of the material during a surgical operation. A further disadvantage of DMB is that it is difficult to handle and lacks structural support of allograft.

3.1.5.1.2 Collagen

Collagen is the most abundant extracellular protein in the body and in bone, and has therefore been suggested to be a potentially useful scaffold for tissue engineering. The first collagen-based material to be used in surgery was catgut-sutures, shown to have low immunogenity and to be highly biodegradable, to the extent that it may be reabsorbed before healing is completed.

For cells to survive, divide and differentiate in culture, it is necessary for them to attach to the culture surface. When collagen is used as a substrate for cell culture, cells attach to the collagen via attachment proteins such as fibronectin (Kleinman et al. 1981). It is possible that this cell attachment could direct growth and differentiation.

It is noted that when osteosarcoma cells were grown on a collagen coated surface the proliferation rate and synthesis of osteocalcin increased (Masi et al. 1992). This suggests that collagen may be a potentially useful medium on which to grow bone. However, when MSCs have been cultured within a collagen matrix, they behave similarly to fibroblasts, contracting and reducing the matrix size, as in scar tissue (Awad et al. 2000). Thus it has been suggested that MSCs differentiate into fibroblasts in collagen. Additionally, collagen is viscous in structure and would offer little structural support to a bone defect. For these reasons collagen was not used in these experiments.

3.1.5.1.3 Fibrin

Fibrin is a constituent of clot, formed from fibrinogen and thrombin mixed. As such, it plays a vital role in wound healing, by forming a network for the migration of fibroblasts and acts as a haemostatic barrier. It has also been shown to promote bone healing when injected into an osteotomy site (Schlag & Redl 1988). It has been shown that fibroblasts cultured within a fibrin clot adhere and proliferate (Gandossi et al. 2000). Therefore, fibrin may be a useful scaffold in which to transfer cells into tissue defects.

However, its effects on MSCs are not known and it is possible that, like collagen, fibrin will stimulate fibroblastic differentiation, as it is chemotactic for fibroblasts *in vivo*. Furthermore, as a fibrin clot is semi-solid in structure, it is more difficult to handle both in the laboratory and potentially so for the surgeon in theatre and also it would not afford structural support to a bone defect.

3.1.5.2 Ceramics

Ceramics include plaster of Paris, a calcium sulphate material, which was first used as a temporary bone substitute in 1892, although with limited success (Peltier 2001). Other ceramics that are suggested to be useful in the tissue engineering of bone are described below.

3.1.5.2.1 Coral

Coral has been investigated as a scaffold for bone growth, as it has a highly interconnected porous structure, high compressive breaking strength and is rapidly biodegraded. Calcium carbonate coral ceramic is derived from the exoskeleton of coral porites and has been shown to support growth and differentiation of marrow derived MSCs on the addition of dexamethasone *in vitro* (Petite et al. 1996).

However, when coral ceramics were compared to hydroxyapatite (HA), both loaded with bone marrow cells and implanted subcutaneously into synergic rats, bone bonded directly to the HA, but there was less ingrowth of bone into the coral and an immune reaction with phagocytosis of the coral was also observed (Ohgushi et al. 1992). This

reaction could represent a foreign body reaction to coral resulting in reduced biocompatibility.

3.1.5.2.2 Bioglass

Bioglass is another calcium phosphate-based ceramic that has been studied for potential bone bonding uses in dental surgery, where it has been shown to be osteoconductive for implanted osteoblasts (Piattelli et al. 2000; Xymos et al. 2000).

3.1.5.2.3 Biphasic ceramic

A mixture of HA and tricalcium phosphate (TCP) is known as biphasic ceramic. Both HA and TCP support the growth of cells *in vitro* (Uchida et al. 1987) and *in vivo* (Shimazaki & Mooney 1985). TCP is absorbed faster than HA once implanted into an animal – at 24 weeks after implantation 46% of TCP has been degraded, compared with 27% of HA (Renooij et al. 1985; Shimazaki & Mooney 1985).

Many studies have examined the osteogenic properties of HA/TCP in research into MSCs (see Chapter 1). When HA/TCP (60/40%) was loaded with bone marrow or isolated MSCs and implanted subcutaneously into an immuno-compromised animal, bone was noted to form within the ceramic (Goshima et al. 1991a; Nakahara et al. 1992). HA/TCP with MSCs have been shown to improve healing of segmental bone defects in dogs (Bruder et al. 1998b). However, *in vitro* MSCs cultured on HA/TCP only differentiated into osteoblasts in the presence of dexamethasone and vitamin D (Toquet et al. 1999).

3.1.5.2.4 Hydroxyapatite (HA)

As calcium phosphate is the major component of inorganic bone, materials based on this have been used as synthetic bone graft substitutes (see Chapter 1). Furthermore, HA ceramics have also been investigated for use in the delivery of osteogenic cells and bioactive factors to sites of bone loss.

HA can be converted from the calcium carbonate exoskeletons of coral by means of a thermal chemical exchange reaction to form a porous scaffold, which resembles cancellous bone (White & Shors 1986). Although physically similar to bone, HA does not possess the same mechanical strength and, therefore, cannot be used for load-

bearing support. However, once bone ingrowth starts the strength of the graft increases (Ohgushi et al. 1989a).

In vivo it has been shown that HA loaded with bone marrow and implanted subcutaneously into a syngenic rat produced bone 2 weeks after implantation (Inoue et al. 1997; Yoshikawa et al. 1992) and that there was no inflammatory response to the implant, confirming its biocompatibility (Okumura et al. 1991). The same results were obtained when cultured bone marrow derived MSCs were similarly implanted on HA (Yoshikawa et al. 2000).

When MSCs were previously cultured with dexamethasone, increased bone formation was observed 1 week after subcutaneously implantation (Yoshikawa et al. 1996). Without the influence of HA, bone only formed when MSCs were cultured with dexamethasone, prior to implantation in a diffusion chamber intraperitoneally (Gundle et al. 1995). An explanation for these findings is that dexamethasone conditions MSCs for osteoblastic differentiation, possibly by differentiating them into committed osteoblast precursor cells prior to implantation, resulting in more rapid bone formation on HA and bone formation where it otherwise would not occur, such as in an intraperitoneal diffusion chamber (Haynesworth et al. 1992b). These results indicate that MSCs on HA form bone when influenced by other factors such as growth factors *in vivo* or dexamethasone *in vitro*.

3.1.6 Effect of surfaces on cells

It has been established that certain properties of materials can affect the proliferation, metabolism, attachment, matrix synthesis and differentiation of cells grown on them. The response by cells varies with cell maturity and can be altered by conditions. The key properties of materials that are known to affect cells are:

- Chemical composition
- Surface energy
- Surface roughness
- Surface topography

Chemical composition affects the absorption of factors from fluid into the surface altering it and thus the cellular response. For example, if fibronectin binds to the surface, it mediates cell attachment and HA coated with fibronectin has been shown to increase MSCs retention in the ceramic (Dennis et al. 1992). A detrimental effect of this chemical conditioning of the material surface by extracellular fluid is leaching of ions from metal implants, where the degree of corrosion is proportional to tissue response (French et al. 1984).

Surface energy relates to the composition of the material as its energy affects the rate of factor absorption and wetability of the scaffold. Surface roughness has also been shown to effect cell behaviour. Bone has been observed to form on rough-surfaced porous HA without cell loading, but not smooth walled HA, when cells were implanted intramuscularly in dogs (Yuan et al. 1999). Therefore, surface roughness appears to have a significant effect on the formation of bone, with rough surfaces tending to promote bone formation, whereas smooth surfaces increase the formation of fibrous tissue. This may be the result of increased protein attachment to the larger surface area of a rough material.

Surface topography, a grooved surface has been shown to allow condensations of osteoblasts to collect resulting in bone formation *in vitro* (Gray et al. 1996; Gray 1998). This effect may be due to osteoblastic stimulation, as the grooves may imitate osteoclast cutting-channels formed in matrix by osteoclasts, which is followed by osteoblastic matrix synthesis in the process of bone turnover.

It is also noted that HA with a porous surface, increases bone formation (Ripamonti et al. 1992); (Yamasaki & Saki 1992) and this may also be due to osteoblasts collecting in the pits. Furthermore, the size of the micro-pores seems to have an effect on the differentiation of MSCs, with a smaller pore size (50-200μm) increasing bone induction in ceramics *in vivo*, to a greater extent than 400μm-500μm pores (Eggli et al. 1988; Ripamonti et al. 1992). As the smaller pores also correspond to the diameter of osteoclast cutting-channels, which are about 50-100μm in diameter, these smaller pores may mediate increased osteoblastic differentiation and bone matrix production, as seen in living bone.

A further explanation for the reaction between osteoblasts and a porous surface may be that the pore size reflects that within cortical bone, also known as the Haversian canaliculi-lacunae system. These pores are much smaller than in trabecular bone, with the lacunae measuring on average 12 x 24 μm and the canuliculi are even smaller (Qin et al. 1999).

3.1.7 Review of scaffold type to be used in this study

As illustrated, both HA and HA/TCP are potentially useful scaffolds for bone regeneration. However, as the rate of bone induction was greater in the HA ceramic when loaded with bone marrow and implanted subcutaneously in a rat (Eggli et al. 1988).

Bone has been shown to bond directly to calcium phosphate ceramic *in vivo*, demonstrating its osteoconductivity (Okumura et al. 1997). Additionally, HA coating on metal prosthetic implants is known to stimulate osseointegration of the prosthesis into bone and it is used therapeutically for this effect (Furlong & Osborn 1991). HA has also been shown to be bioactive *in vivo* and it fulfils a number of the property requirements for tissue engineering of bone: biocompatibility, osteoconductivity and porosity. Hence, the effect of using HA without the influence of growth factors, on the differentiation of MSCs *in vitro* will be investigated in this chapter.

In my thesis, the HA scaffold used had interconnecting macro-pores, similar in structure to that of trabecular bone. This structure would also allow the growth of cells throughout the scaffold and the ingrowth of capillaries into the construct following implantation into a patient.

As noted above, a rough, micro-porous surface of HA has been shown to induce bone formation. The micro-pores in the HA scaffold used in my thesis were of a similar size to those of the canaliculi-lacunae, measuring on average 10μm, with the intention that this may also increase osteoblastic activity.

3.1.8 Bioactive factors

Bioactive factors that have been considered are those such as BMPs, which were first discovered in demineralised bone matrix (Urist 1965). However, it has been shown that doses many times greater than physiological levels are needed to exert an osteogenic effect clinically (Boyne et al. 1997; Howell et al. 2002). The use of bovine BMP would cause concern due to the possible transmission of prions and the long-term effects of administering these doses of rhBMP to patients also still needs to be assessed fully.

Although the proportions of multiple growth factors present *in vivo* cannot easily be re-created *in vitro*, following implantation of the tissue engineered construct, the cells would be subject to a physiological environment. Hence, if tissue can be generated *in vitro* without bioactive factors, maturation will increase on implantation *in vivo*. For these reasons, in my study, the differentiation of MSCs into osteoblasts and production of extracellular bone matrix on HA was tested without the use of bioactive factors.

3.1.9 In this chapter

The aim of this chapter was to investigate whether MSCs could grow on HA and whether this surface would stimulate cellular differentiation down the osteogenic lineage. Thereby, committed, lineage specific osteoblastic cells could be tissue engineered on HA scaffolds for potential implantation aiding rapid bone repair.

As discussed above, for MSCs to be used in the tissue engineering of bone, a suitable carrier needs to be investigated. There is some evidence from the *in vivo* studies described above that HA may encourage differentiation of MSCs, however this has not been investigated *in vitro*, without the effect of osteogenic factors that are present *in vivo*. Thus, this will be investigated in this chapter. Additionally, for HA to be a useful carrier of MSCs, the cells need to be able to grow on the HA scaffold and so proliferation of cells was tested using Alamar Blue and DNA assays.

To study the effect of HA, bone marrow isolated MSCs were cultured on an HA surface and compared with cells grown on culture plastic *in vitro*. These two surfaces were used to measure whether growth on an HA surface was associated with an

increase in production of osteoblastic proteins. For MSCs to be used for tissue engineering of bone, a structure that can be used to fill bone defects is necessary, thus MSCs were also cultured on porous HA scaffold and cell proliferation and osteoblastic differentiation were both assessed. As this 3-d structure may affect cellular behaviour these results were compared to a control plastic scaffold with a similar structure.

Evidence for osteoblastic differentiation of the cultured cells was observed under light microscopy and scanning electron microscopy (SEM), as described in Chapter 2. Transmission electron microscopy (TEM) was used to assess the ultrastructure of the cells and extracellular matrix production on the scaffold. Biochemical assays were used to measure the levels of osteoblastic proteins produced by the cells, as detailed in Chapter 2.

3.1.10 Hypothesis

The hypotheses tested this chapter were:

1. MSCs isolated from human bone marrow will grow on an HA surface or scaffold *in vitro*;
2. MSCs differentiate into osteoblasts without the influence of OS when cultured on either HA discs or porous HA scaffolds *in vitro*.

3.2 MATERIALS and METHODS

3.2.1 Materials

General

Cell culture materials and assays as listed in Chapter 2

Alamar blue assay (Serotec BUF012B)

Hydroxyapatite porous pieces (Plasma Biotal): cylinders 12mm height x 10mm diameter, sterilised by gamma irradiation

Titanium discs 10mm diameter coated with HA (Plasma Biotal), sterilised by gamma irradiation

SEM as listed in Chapter 2

TEM

Propylene oxide (Agar Scientific R1080)

Hard resin:

Vinyl cyclohexanedioxide 20g (Agar Scientific R1047)

Noneryl succinic anhydride 52g (Agar Scientific R1055)

Diglycidylether of polypropyleneglycerol 8g (Agar Scientific R1073)

Dimethylaminoethanol 0.8g (Agar Scientific R1067)

3.2.2 MSC culture

Twelve human bone marrow aspirates were taken from patients after informed consent, from which the MSCs were separated and cultured in monolayer, as previously described in Chapter 1. The MSCs were again observed to form colonies of cells in culture. Once the primary cultures became confluent, the cells were trypsinised and expanded until passage 4, when enough cells from each of the aspirates had been grown to seed onto 2-dimensional (2-d) HA discs and 2-d plastic controls, as well as 3-d HA scaffolds and 3-d plastic controls. All procedures were carried out in the laminar flow cell culture hood using a no-touch technique and cells were cultured at 37°C and 5% CO_2 throughout.

In summary, to assess the effect of a HA surface on proliferation and differentiation, MSCs were initially cultured on HA coated discs and compared with MSCs grown on a plastic control. Differentiation of MSCs into osteoblasts was examined by observing and measuring levels of osteoblastic proteins produced over time for cells on both HA and control surfaces.

3.2.3 2-Dimensional Hydroxyapatite Discs

Firstly, HA discs, sterilised by gamma irradiation, were each seeded with 25,000 MSCs in a volume of 50µl of medium, onto the centre of the disc. An identical method was used to seed the control cells onto tissue plastic in a 24 well plate. After 2 hours of incubation and before the cells dried out, each well was flooded with 2ml of medium, which was then changed every other day. Standard medium (content as detailed in Chapter 2) was used throughout the experiments of this chapter, unless otherwise stated. This method was used to assemble discs and controls for the proliferation experiments, differentiation assays and for SEM.

3.2.3.1 Measure of Cellular proliferation

3.2.3.1.1 Levels of DNA

HA discs and six plastic controls were each seeded with 25,000 MSCs and the DNA content was measured, after 5, 10 and 15 days in culture, using DNA Hoeschst assay as described in Chapter 2 Method. This was repeated with MSCs separated from six patients' aspirates. The DNA levels were then compared statically between the two surfaces as a further marker of cell growth.

3.2.3.1.2 Absorbance of Alamar blue assay

The cellular proliferation rate was measured using Alamar blue assay, adapted from published methods (Nakayama et al. 1997; Nikolaychik et al. 1996; Nociari et al. 1998).

Alamar blue assay was used to compare the proliferation rate between MSCs seeded onto HA discs and the control. Six HA discs and six plastic disc controls were each seeded with 25,000 MSCs. These sample groups were repeated for three patients' marrow aspirates giving a total of 18 test discs for each group. The cells were cultured for 15 days, with Alamar absorbance being measured every 5 days.

To measure Alamar blue absorbance, the medium was removed from the cells and replaced with 0.5ml of 10% Alamar blue assay in phenol-free medium. A similar amount of Alamar blue was also placed in an empty well to act as a control. After the cells had been incubated for 3 hours, 2 repeats of 100µl of Alamar blue were removed from each well and the absorbance was measured at 570nm for each sample and control. The control absorbance (background) was subtracted from the result of each sample. The cells were then washed twice with phosphate buffered saline, fresh medium was added and the cells continued in culture.

3.2.3.2 Observational study of cellular differentiation on HA

3.2.3.2.1 Under scanning electron microscopy (SEM)

In a separate study, the difference in morphology of MSCs grown on HA discs, therminox discs in standard medium (negative control for osteoblastic differentiation) and therminox discs in OS (positive control, OS constituents as described in Chapter 2) was observed under SEM. For this study, MSCs were cultured on 3 discs for each of the culture periods: 1, 7 and 14 days, for each of the conditions noted above. This was repeated for MSCs derived from 2 patients.

The cells were seeded using the method described above, although a cell density of 20,000 cells was used. Following this, discs were processed for SEM (see Chapter 2 Method). The cellular morphology of MSCs on HA was assessed after 1, 7 and 14 days in culture and compared with MSCs cultured on therminox discs, in standard and OS medium.

3.2.3.3 Measure of protein assays, an indicator of osteoblastic differentiation

3.2.3.3.1 ALP

The amount of ALP produced by the cells was used as a measure of osteoblastic differentiation, using a biochemical assay for p-nitrophenol cleaved from p-nitrophenol phosphate (see Chapter 2 Method). For each of the protein assays 25,000 MSCs from six patients were seeded onto each of six HA discs and six plastic controls, for each time point measured. Cells were cultured for 5, 10, 15 and 30 days, at which points the level of ALP and total DNA content of each sample was measured (see Chapter 2

Method). The amount of ALP per disc was standardised for a variation in the increase in cell number over the culture period, by dividing the ALP measurement of each sample by total DNA content, thus allowing comparisons to be made between ALP production on HA and control discs at each time point.

The experiment was repeated using eight HA discs with 250,000 cells per disc and ALP and DNA levels were measured in the same way after 14 and 28 days.

3.2.3.3.2 Osteopontin

Osteoblastic differentiation was also assessed by measuring the production of osteopontin. For this study marrow aspirates from six patients were used as 250,000 MSCs were cultured on six HA discs and six plastic controls for 14 days and this was repeated on six further HA discs for 28 days. The level of osteopontin protein was measured using an enzyme immunometric assay (see Chapter 2 Method) and, as before, the readings were divided by total DNA content to allow comparison.

3.2.3.3.3 Osteocalcin

The osteocalcin assay detailed in Chapter 2 was used as a further marker of osteoblastic differentiation, by comparing protein levels between cells grown on HA discs and controls when 250,000 cells were cultured for 14 days. This was repeated with five patients' aspirates. DNA content was again measured for standardisation.

3.2.4 3-Dimensional Porous Hydroxyapatite Scaffold

The second part of this study investigated the effect of growing MSCs on porous 3-d HA scaffolds. The scaffolds were cylindrical in shape with a height of 12mm and diameter of 10mm and had a 70% void volume. They had an interconnected porous structure of 1mm macroscopic pores and 10μm microscopic pores. For each of the experiments, cells were seeded at a density of $1x10^6$ cells per scaffold in 0.5ml of standard medium. The cells were incubated for 2 hours and then flooded with medium, which allowed the cells to start to attach to the scaffold without the medium evaporating dry, avoiding cell lysis.

3.2.4.1 Measurement of cellular proliferation

3.2.4.1.1 Alamar blue assay

Marrow aspirates were taken from eight patients and an alamar blue assay was performed on eight sets of HA scaffolds cultured with $1x10^6$ cells for 5, 10, and 15 days, as described above. Alamar blue assay was similarly performed on MSCs grown on plastic scaffolds, which had been seeded with the same number of cells for the same culture period, as a comparison.

3.2.4.1.2 DNA levels

In the same way as for HA discs, the total DNA content of cells cultured on six HA scaffolds seeded with $1x10^6$ MSCs was measured after 5, 10 and 15 days.

3.2.4.2 Observational study of the cells on HA scaffolds

3.2.4.2.1 Light Microscopy

In a separate study, after 5 days in culture, the HA scaffolds were stained with toluidine blue, by covering the scaffold in the stain for 3 minutes before washing with distilled water. This stained the cells blue, allowing them to be visualised under light microscopy using a high-powered halogen light source.

3.2.4.2.2 SEM

In a further study, an HA scaffold was cultured with MSCs for each of 5, 7 and14 days. These samples were repeated with cells from each of 3 patients. Thus, after each time-point, 3 scaffolds were processed for SEM, using the method described in Chapter 2. The morphology of the cells on the HA scaffolds was observed under SEM and compared between the 3 time-points.

3.2.4.2.3 Transmission Electron Microscopy (TEM)

MSCs from 3 patients were each cultured on 3 separate HA scaffolds for 28 days after which they were processed for TEM.

The initial processing for TEM was as for SEM (see Chapter 2 Method), until the stage of drying the samples in alcohol, where for TEM the graded stages started at 70% alcohol and increased to 100% ethyl alcohol. Following this the samples were dried in

propylene oxide for 10 minutes before embedding in resin. Hard resin was made using the ingredients listed in the Materials section. Each HA scaffold was placed in a container full of resin and was left in a vacuum chamber overnight to increase resin penetration. Sections measuring 1mm were cut using a peripheral diamond cutter. Areas containing scaffold were re-embedded in resin. Thin sections were then cut using an ultra-microtome with a diamond blade, placed on copper grids and observed under TEM (Philips CM12).

3.2.4.3 Protein assays of osteoblastic differentiation

The differentiation of MSCs into osteoblasts was also quantified by the degree of protein production by the cells. An HA scaffold was seeded with $1x10^6$ cells for each of eight patients' MSCs aspirates. This was repeated for each culture period and test, and biochemical assays were used to measure ALP and osteocalcin production.

3.2.4.3.1 ALP

The production of ALP by cells was measured after 5, 10 and 15 days in culture (see Chapter 2 Method). The total DNA content was also measured to allow standardisation, as for the HA discs.

3.2.4.3.2 Osteocalcin

Osteocalcin production by cells cultured on the 8 sets of HA scaffolds was measured after 15 days in culture. Osteocalcin levels were measured using a radioimmunoassay, as described in Chapter 2 Method. The total DNA content of the samples was measured to standardise the results, as before.

3.2.5 Use of 3-Dimensional Porous Plastic Polymer control

A 3-d control with the same shape as the porous HA scaffolds but in an inert material was used as a comparison. As polyurethane has been used in tissue engineering research as a porous membrane for culture of hepatocytes (Pahernik et al. 2001), it was used as the control (Kurosawa et al. 2000). Porous polyurethane plastic cylinders with the same pore structure and dimensions as the HA scaffolds were seeded with $1x10^6$ MSCs from the same patients and identical culture methods used for the HA (see above) were employed.

In summary, control scaffolds were also stained with toludine blue and observed under light microscopy after 5 days in culture. Again, the proliferation rate was assessed after 5, 10 and 15 days, using Alamar blue assay.

3.2.6 Statistical tests

The results of the protein assays after each time point were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk statistical tests, but as results did not follow normal distributions, non-parametric statistical tests (Mann Whitney U) were applied. A P-value <0.05 was taken to be significant throughout the studies.

3.3 RESULTS

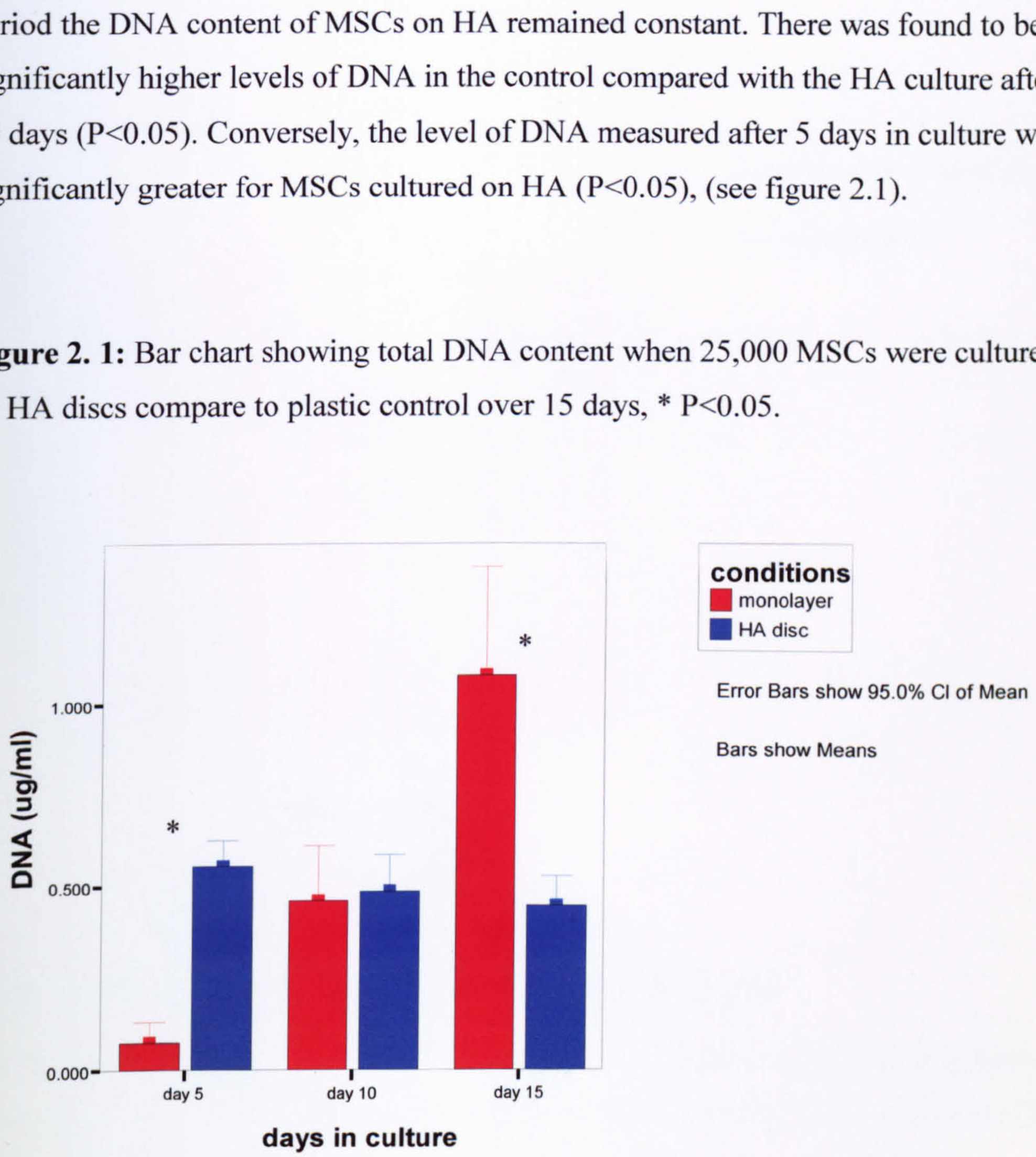
3.3.1 MSCs cultured on 2-Dimensional Hydroxyapatite Discs

3.3.1.1 Assessment of cellular proliferation rate on HA discs

3.3.1.1.1 DNA levels

The total DNA content of MSCs grown over 15 days was measured at 5-day intervals. The amount of DNA from the cultured control cells increased between day 5 and 10 and between day 10 and 15 in culture ($P<0.005$). However, over the same culture period the DNA content of MSCs on HA remained constant. There was found to be significantly higher levels of DNA in the control compared with the HA culture after 15 days ($P<0.05$). Conversely, the level of DNA measured after 5 days in culture was significantly greater for MSCs cultured on HA ($P<0.05$), (see figure 2.1).

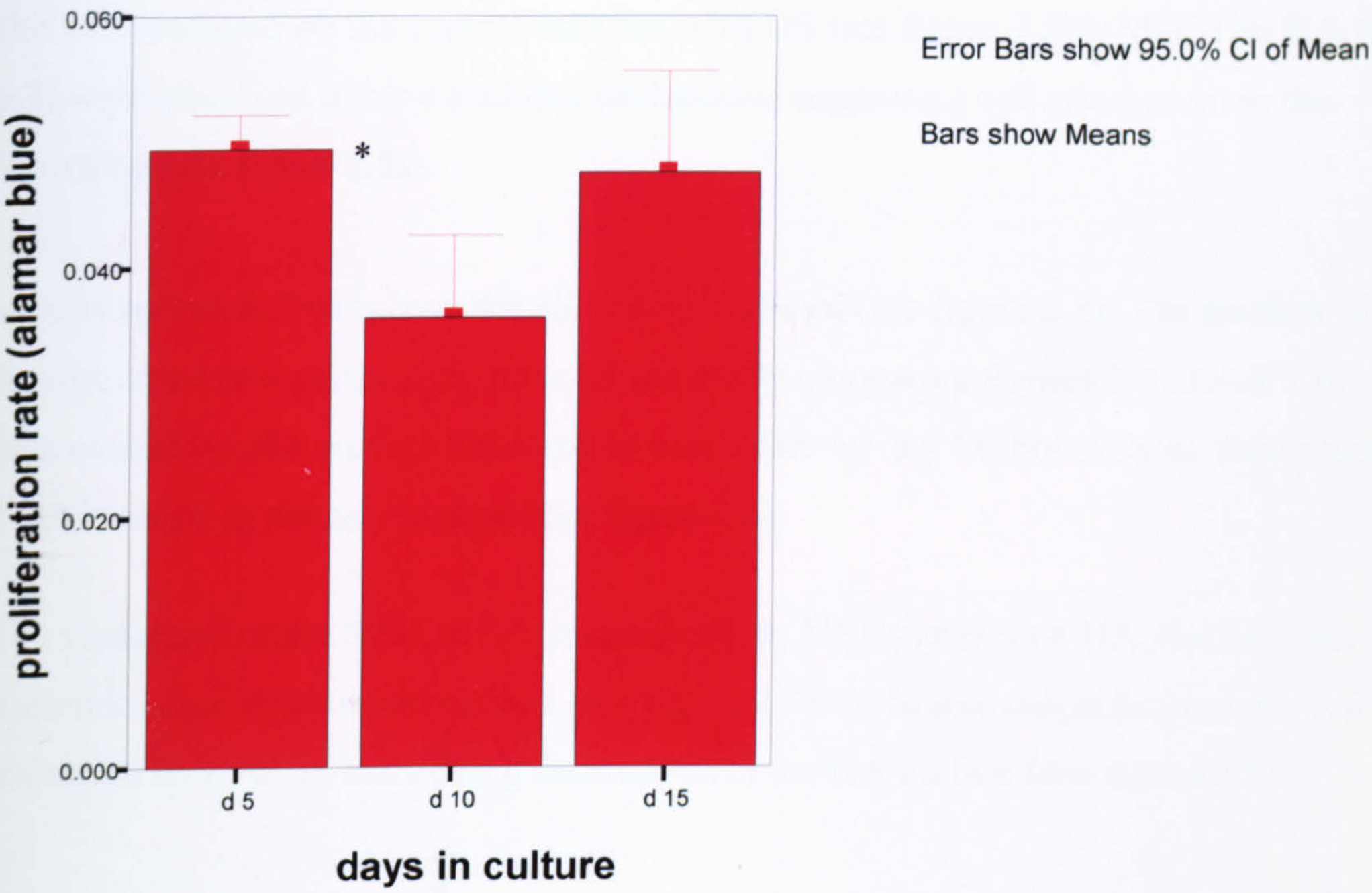
Figure 2. 1: Bar chart showing total DNA content when 25,000 MSCs were cultured on HA discs compare to plastic control over 15 days, * $P<0.05$.



3.3.1.1.2 Alamar blue assay results

The absorbance measured by Alamar blue assay on the HA surface was significantly higher after 5 days compared to 10 days in culture ($P<0.005$). The rate increased again after 15 days in culture, although this was not found to be significant on statistical testing (see figure 2.2).

Figure 2. 2: Bar chart showing proliferation rate of MSCs on HA discs measured by Alamar blue over 15 days, * $P<0.005$.



3.3.1.2 Observational study of MSCs differentiation on HA

The first part of this study examined the response of MSCs to culture on HA discs by comparing cells cultured over 14 days on HA with the control, with and without OS, which stimulates osteoblastic differentiation of MSCs, as shown in Chapter 1.

3.3.1.2.1 SEM Results

SEM was used to compare MSCs over 2 weeks in culture on HA discs with the therminox control with and without OS. As shown in figure 2.3, MSCs in standard medium on therminox were characteristically spindle shaped, whereas the cells in culture with OS started to change shape after 1 day, becoming squarer. However, MSCs in standard medium maintained their fibroblastic morphology over the total culture period (see figures 2.3a, 2.4a, 2.5c & 2.6a).

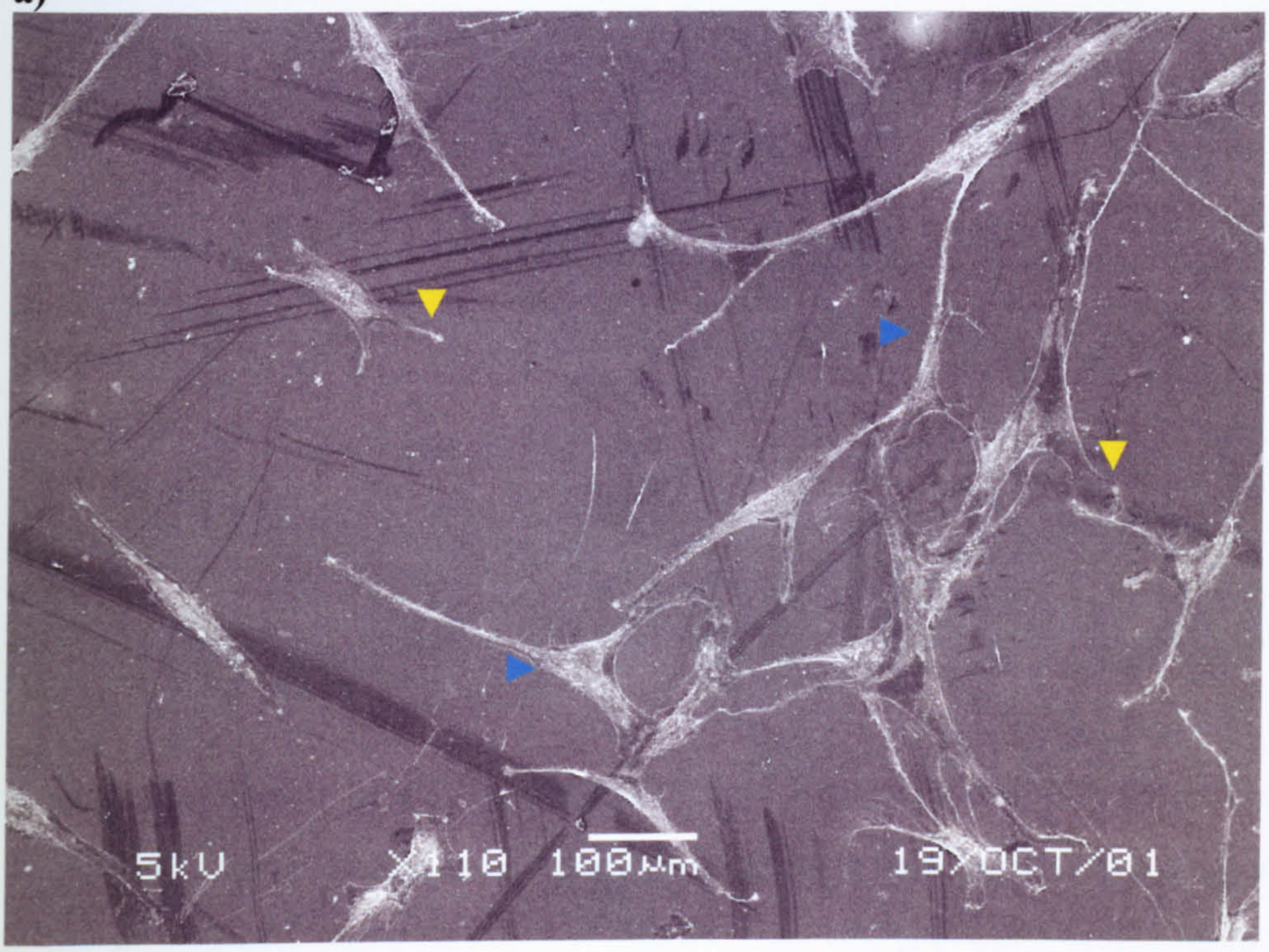
The morphology of the cells cultured on HA discs changed over the culture period, becoming less fibroblastic and more osteoblastic (see figure 2.7) and similar in shape to the cells cultured on the control cultured with OS (see figure 2.5b). MSCs on HA at day 7, were observed to have multiple philapodia, suggesting cell attachment to the HA surface (see figure 2.5a).

The cells spread diffusely over the all culture surfaces (see figure 2.3). The number of cells was noted to increase over time, as shown by comparing figures 2.3, 2.4 & 2.6. MSCs coated the HA surface attaching to each other by day 14, but cells on therminox did not become as densely packed (see figure 2.6).

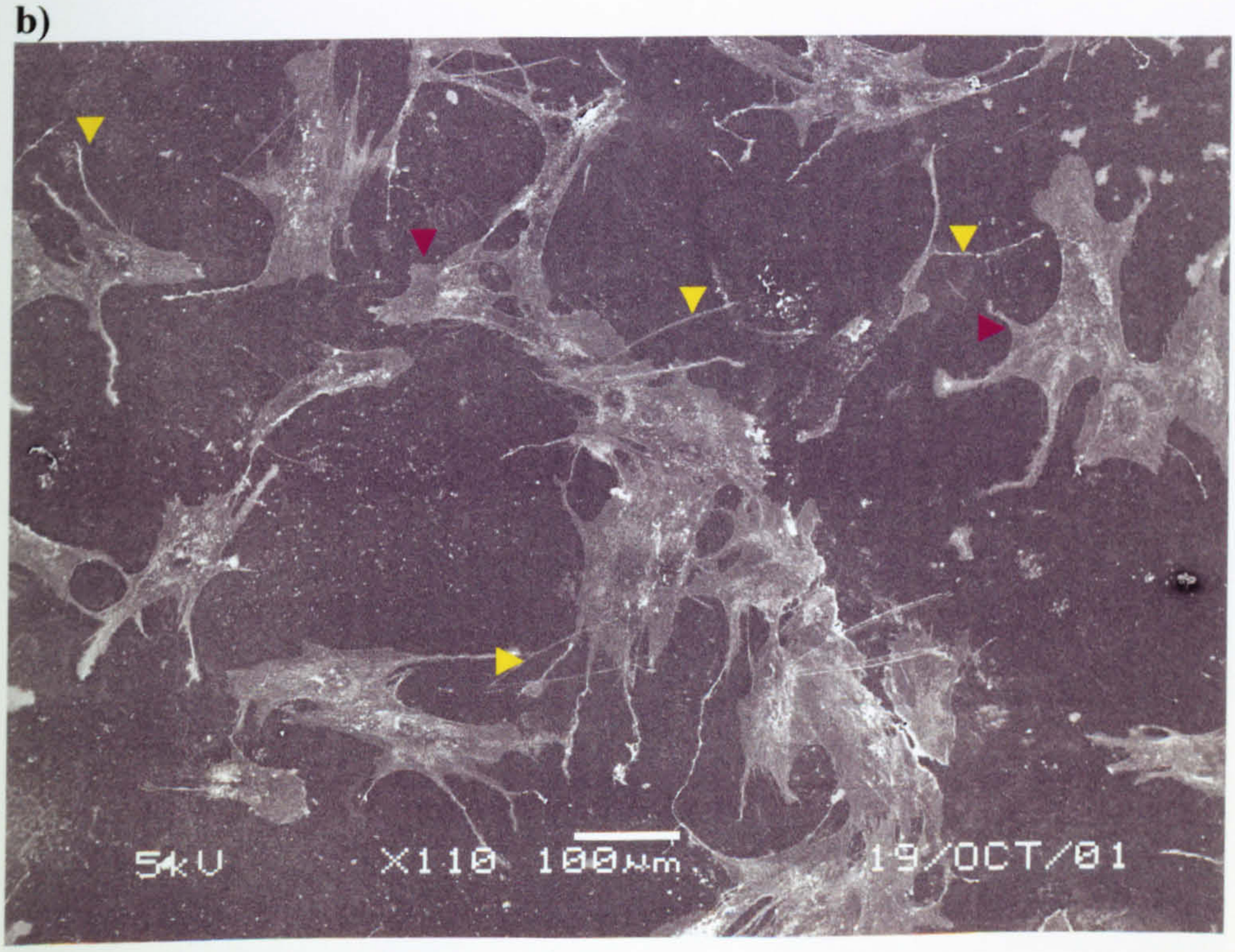
When visualised under SEM at low magnification, MSCs grown on HA discs where less distinct than those on therminox (see figure 2.3). At higher magnification the cell membranes seem to be embedding themselves in the HA surface (see figure 2.8).

Figure 2. 3: Day 1: MSCs cultured on a) therminox in standard conditions, b) therminox with OS and c) HA discs, bar = 100μm. Blue arrows indicate cells with spindle morphology, pink arrows show squarer cell shape and yellow arrows show cell processes attaching to the surface.

a)



b)



c)

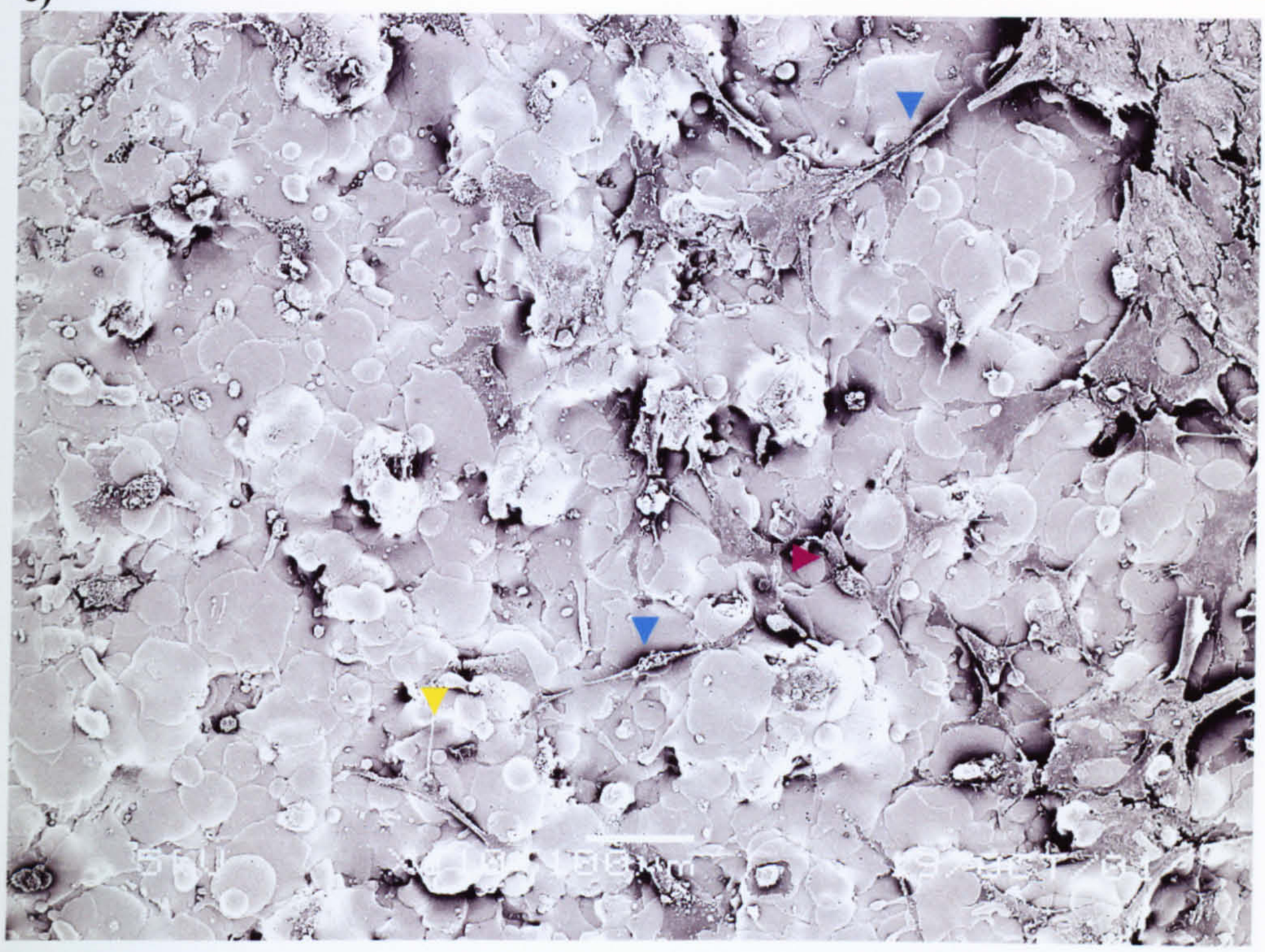
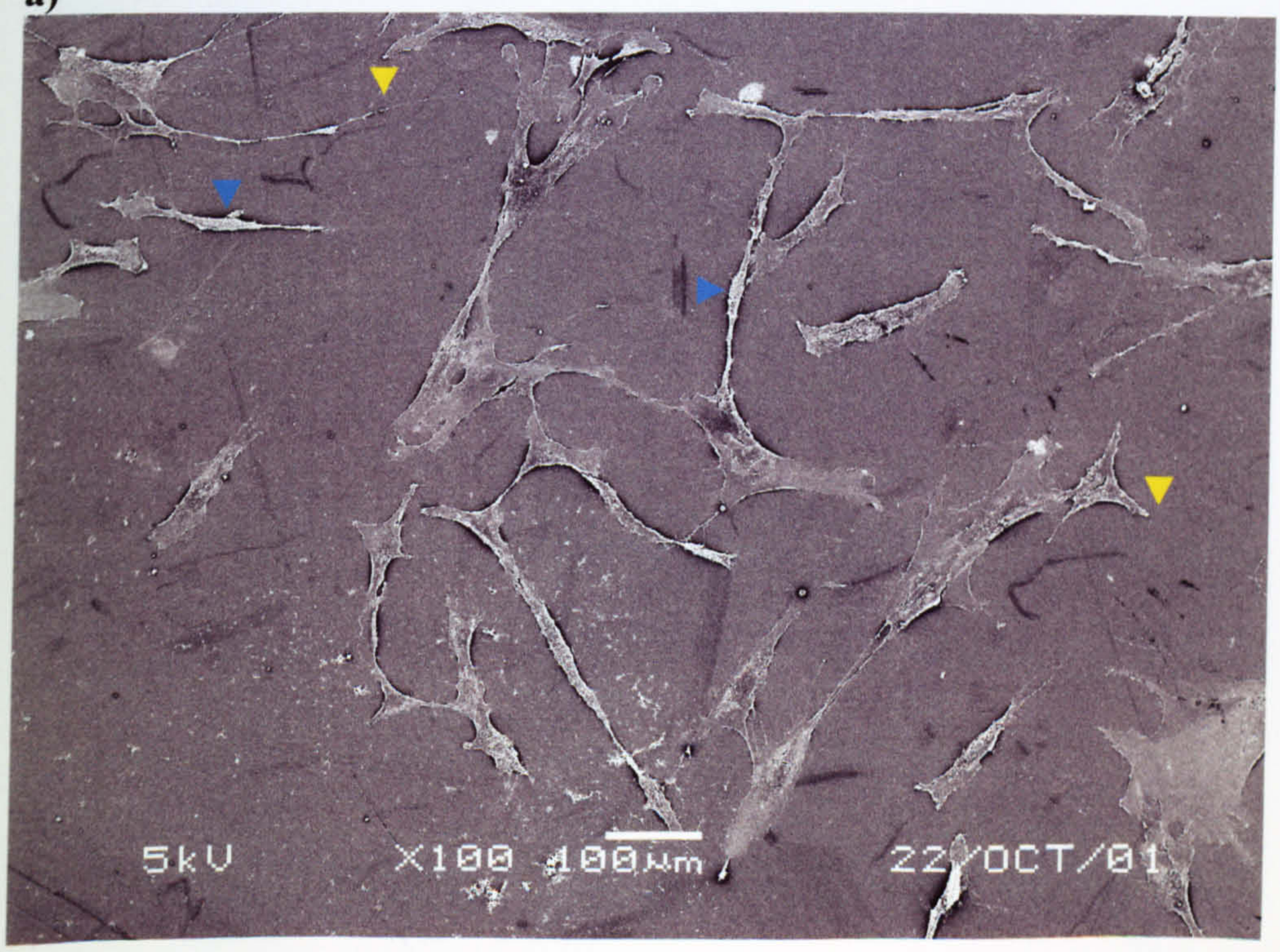
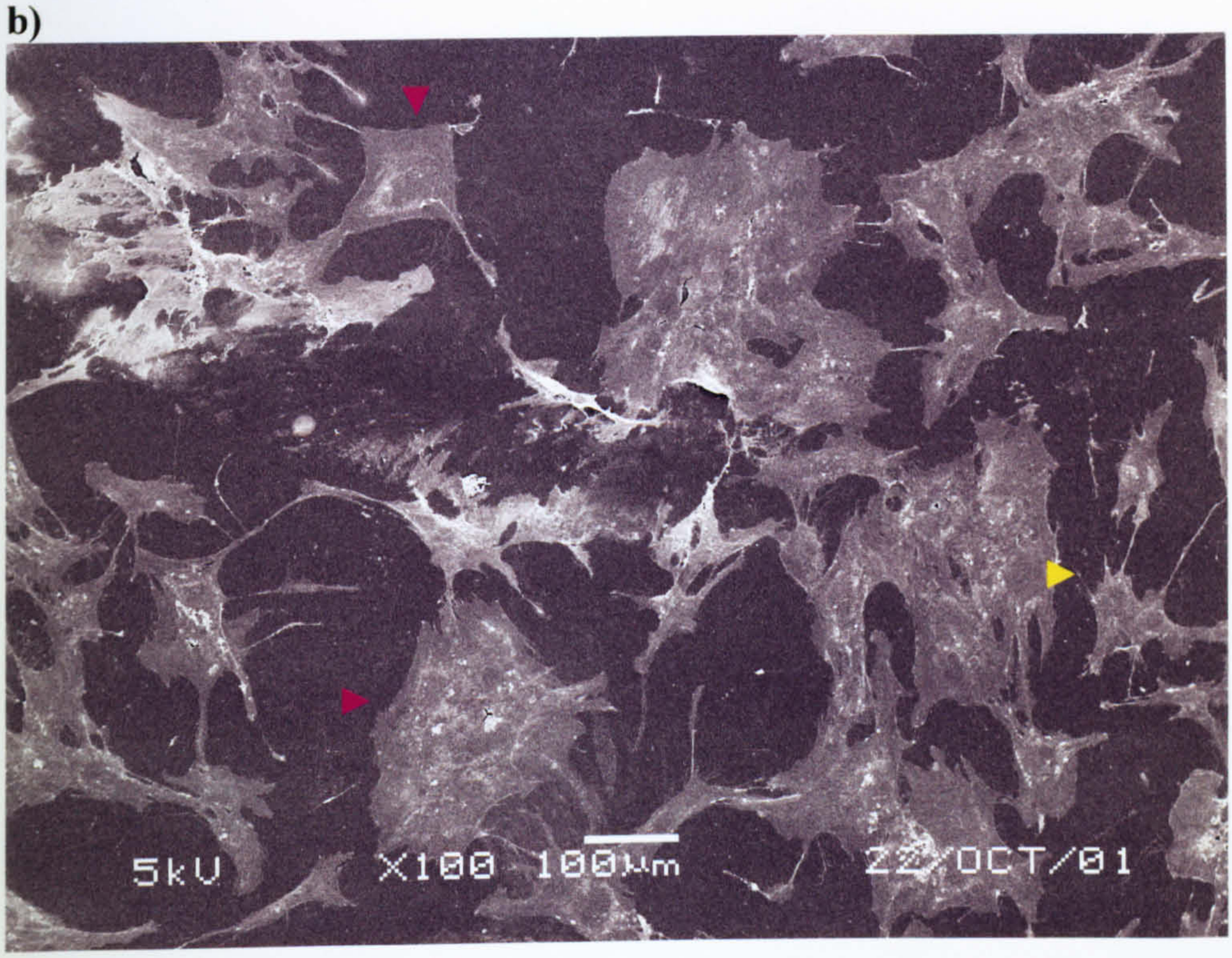


Figure 2. 4: Day 7: MSCs cultured on a) therminox, b) therminox with OS and c) HA discs, bar = 100μm. Blue arrows indicate cells with spindle morphology, pink arrows show squarer cell shape and yellow arrows show cell processes attaching to the surface.

a)



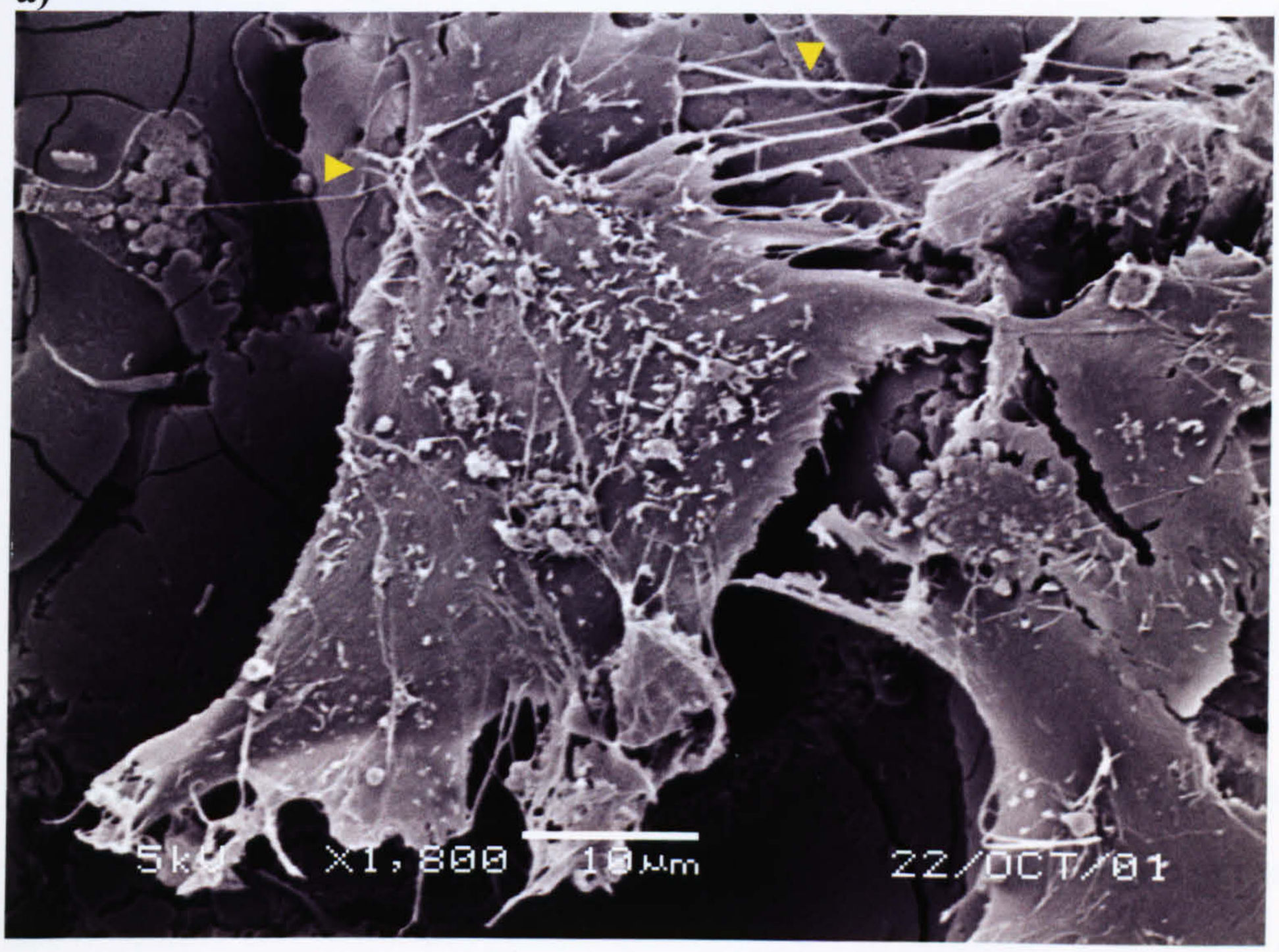
b)



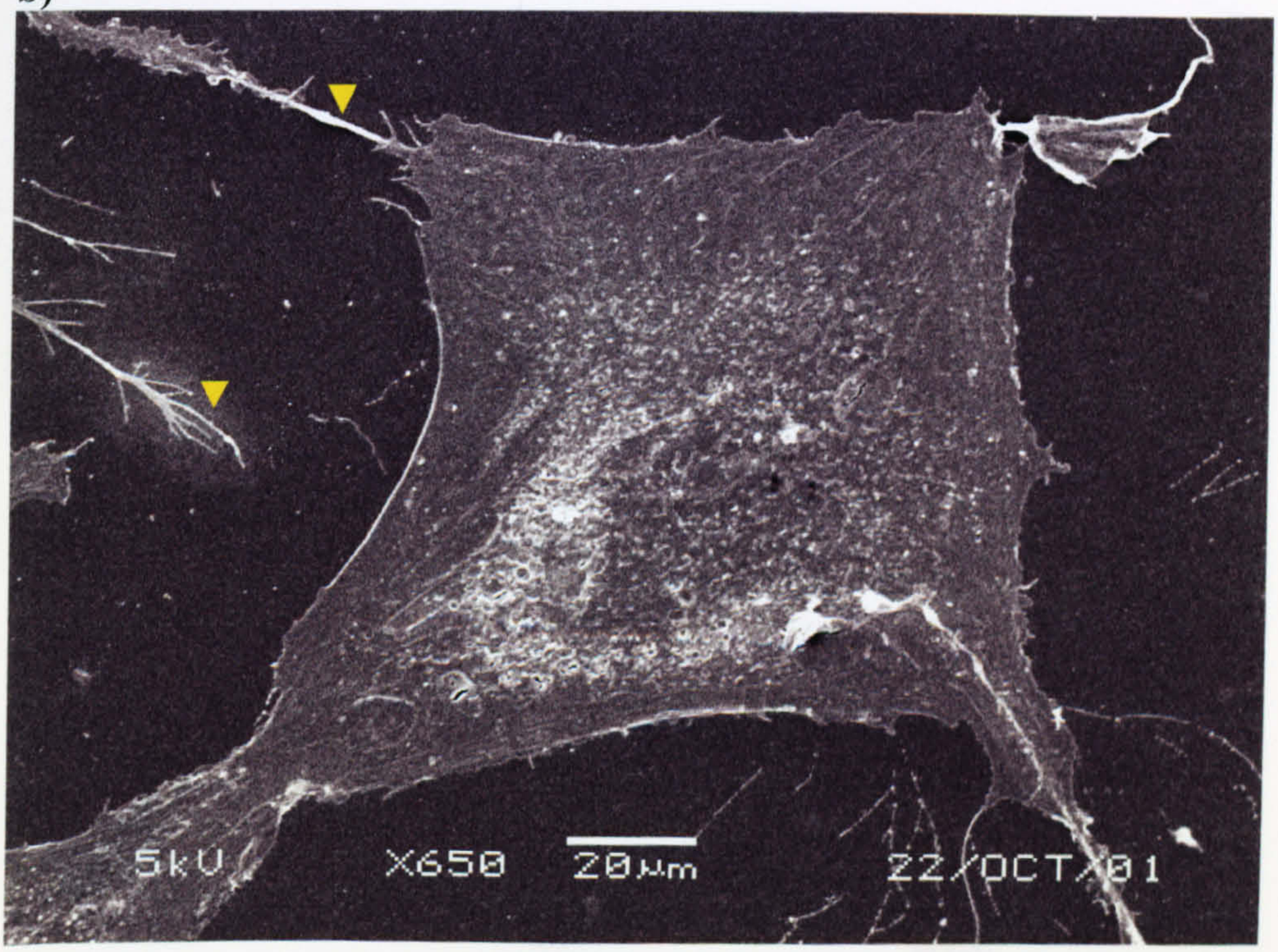
c)

Figure 2. 5: Higher magnification at day 7: SEMs of MSCs cultured on a) HA discs: cuboidal shaped cells with multiple processes yellow arrows, b) therminox with OS: cuboidal shaped c) therminox in standard conditions: spindle shaped cells. Magnification is indicated by bar on each picture.

a)



b)



b)

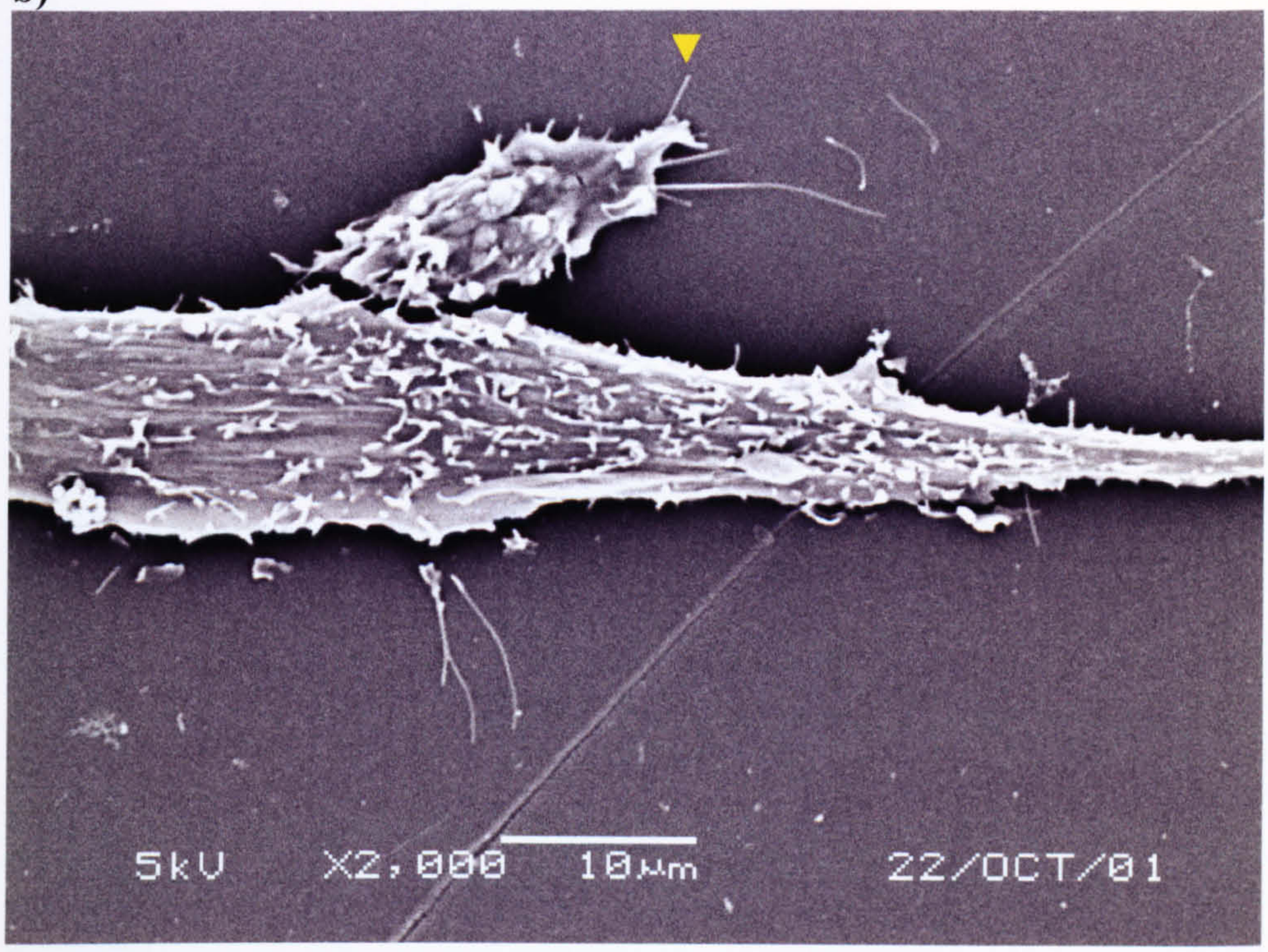
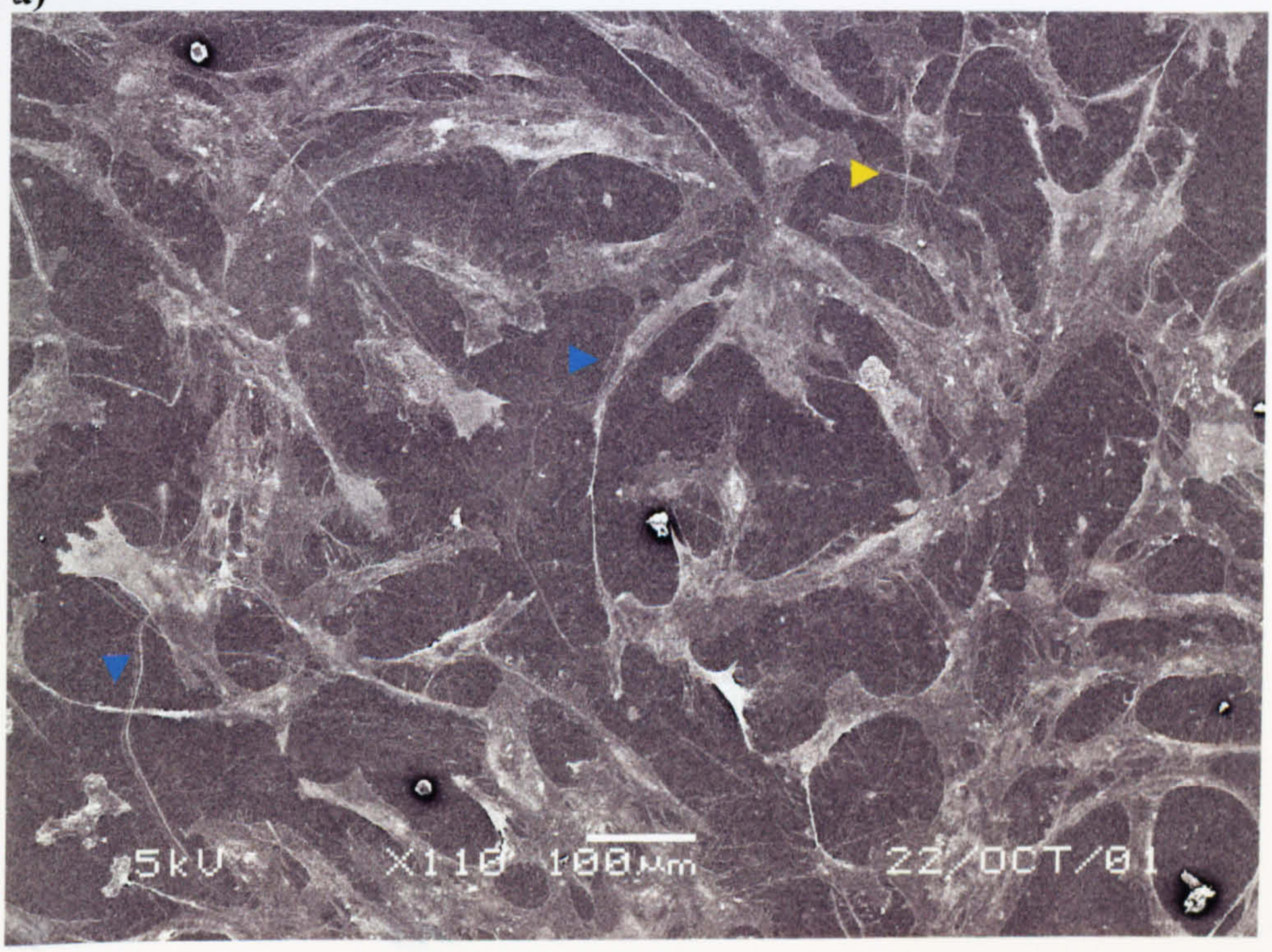
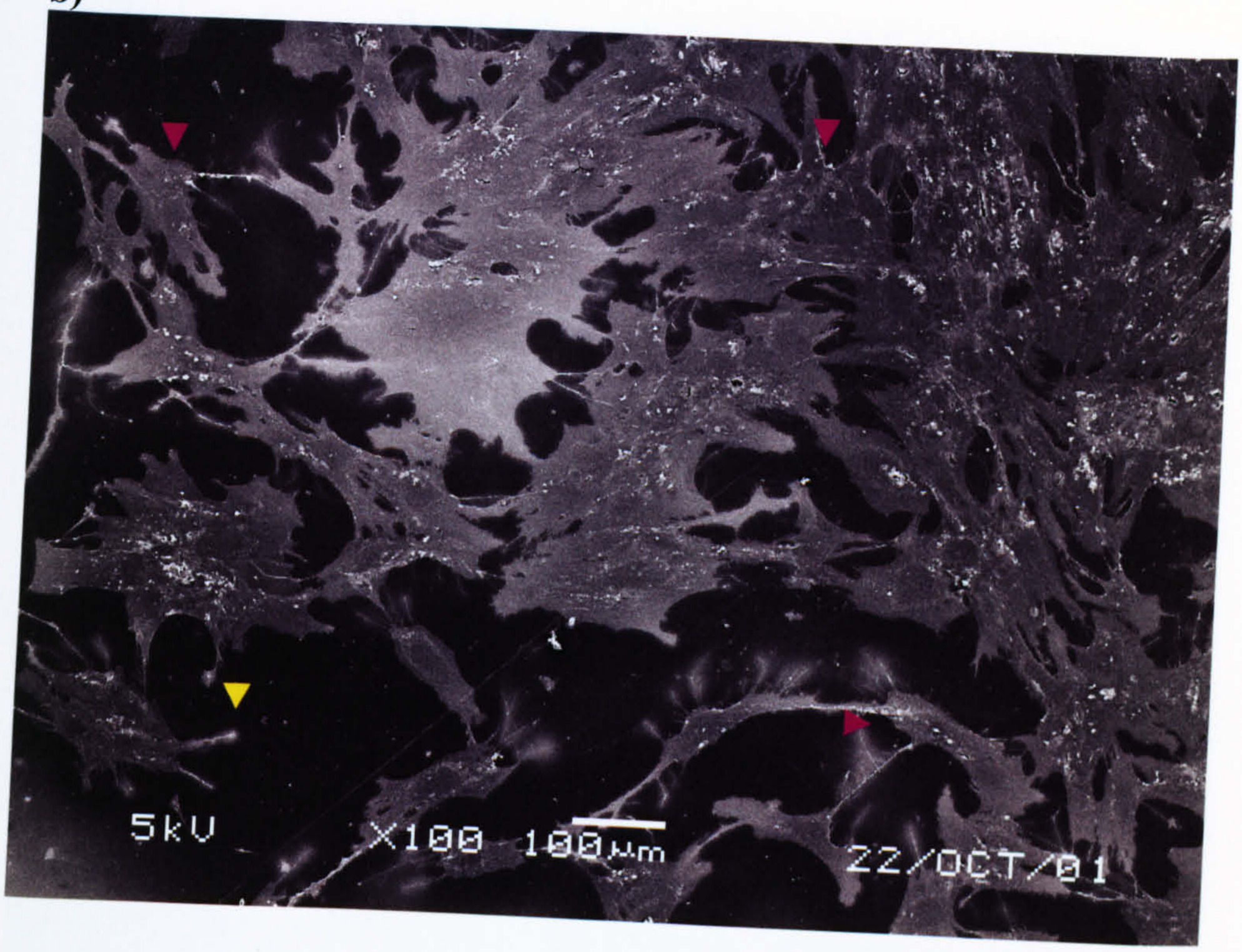


Figure 2. 6: Day 14: MSCs cultured on a) therminox, b) therminox with OS and c) HA discs, bar = 100µm. Blue arrows indicate cells with spindle morphology, pink arrows show squarer cell shape and yellow arrows show cell processes attaching to the surface.

a)



b)



c)

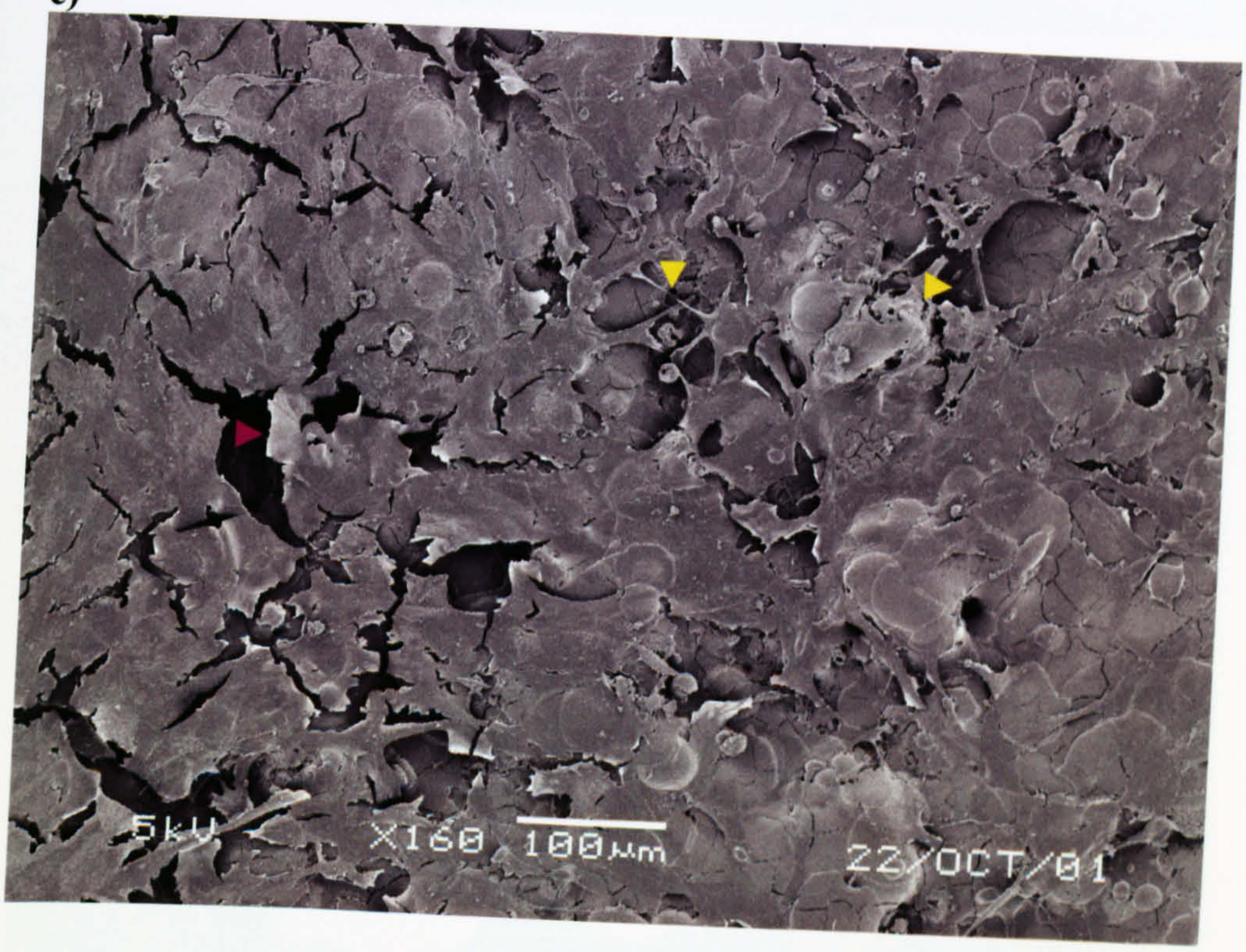
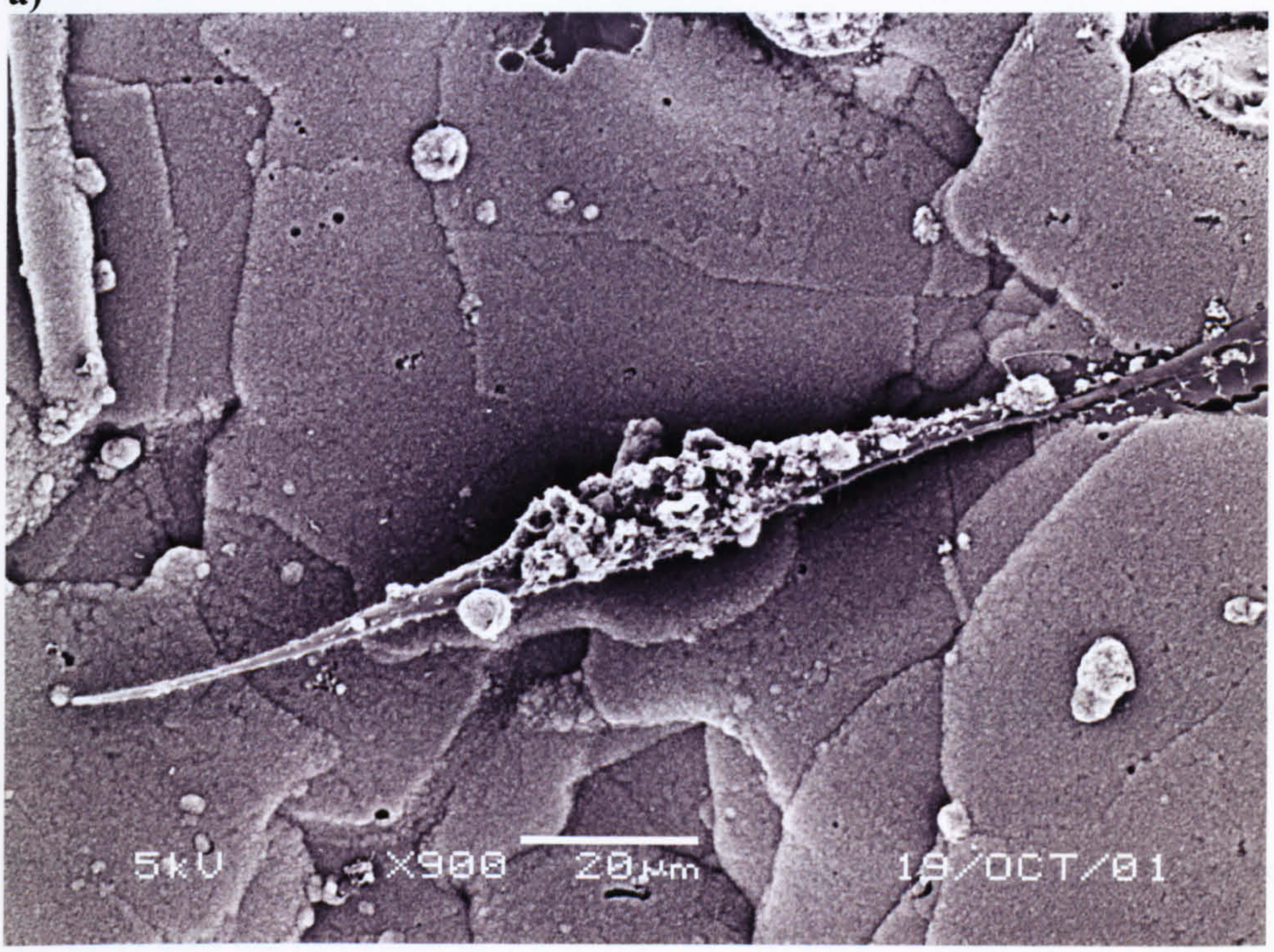
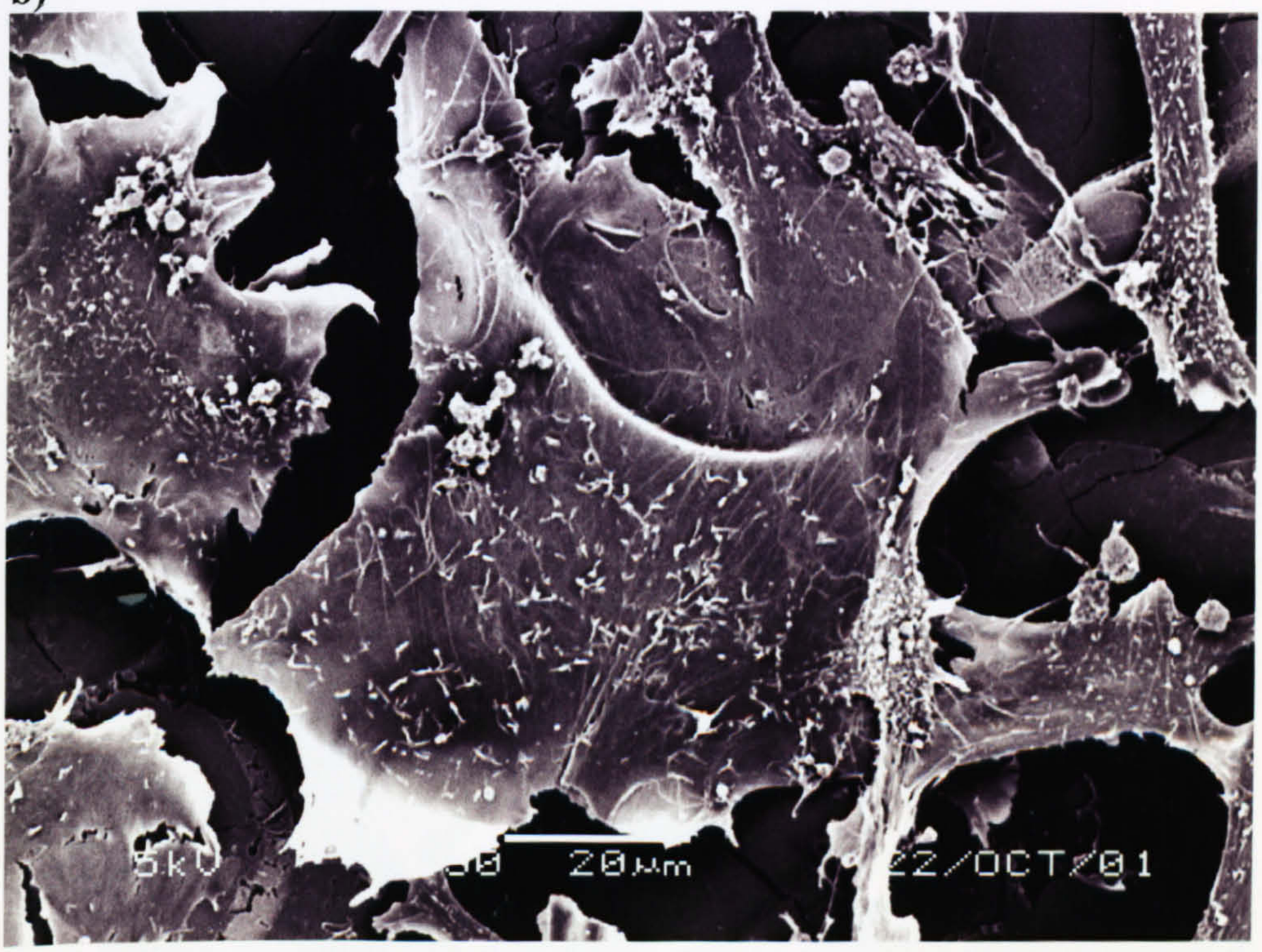


Figure 2. 7: These SEM shows changes in the shape of MSCs cultured on HA discs in standard conditions, after a) 1 day: spindle shape, b) 7 days: cuboidal shape & c) 14 days: dense cell covering. Bars on each picture indicate the level of magnification.

a)



b)



c)

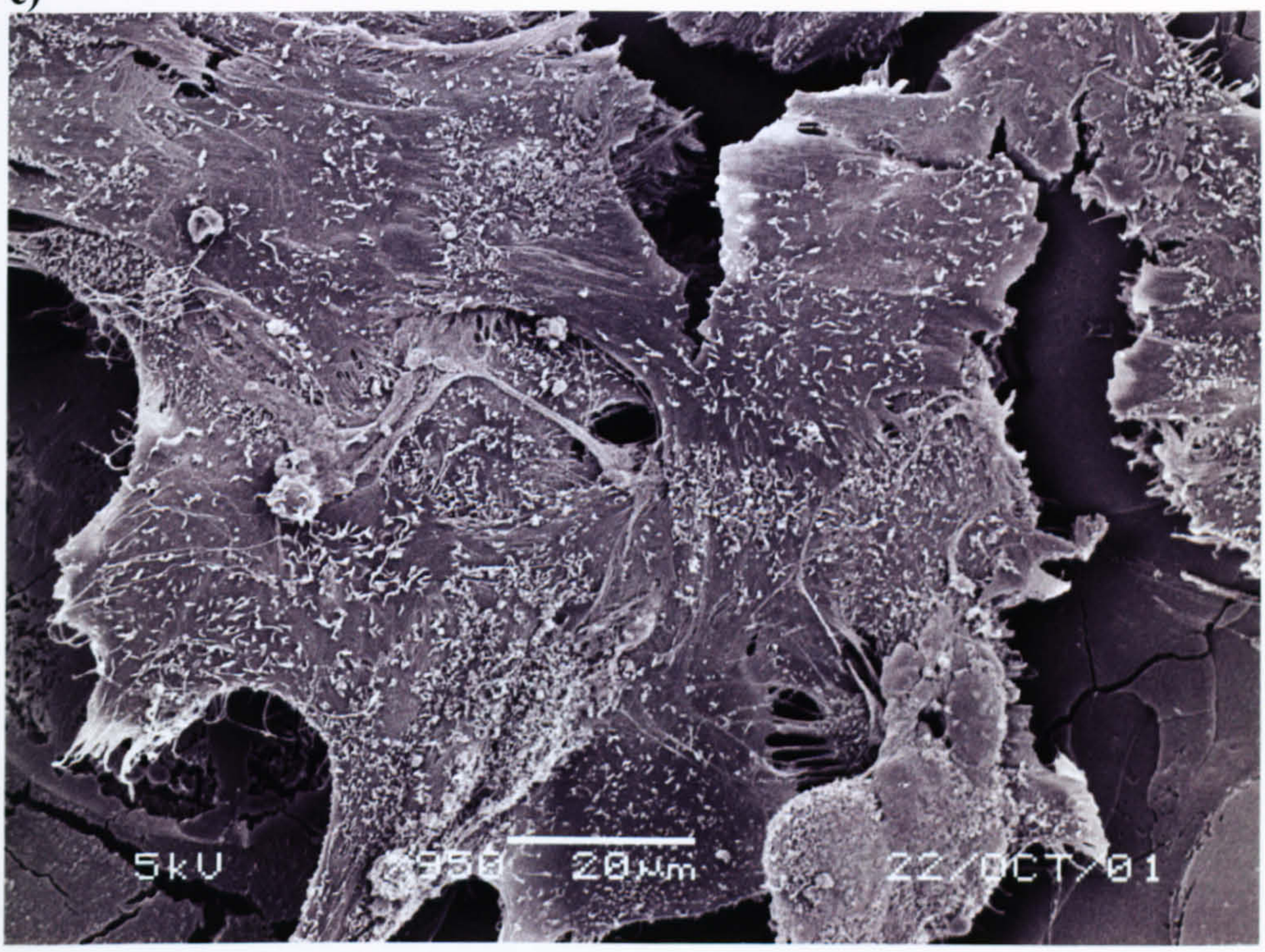
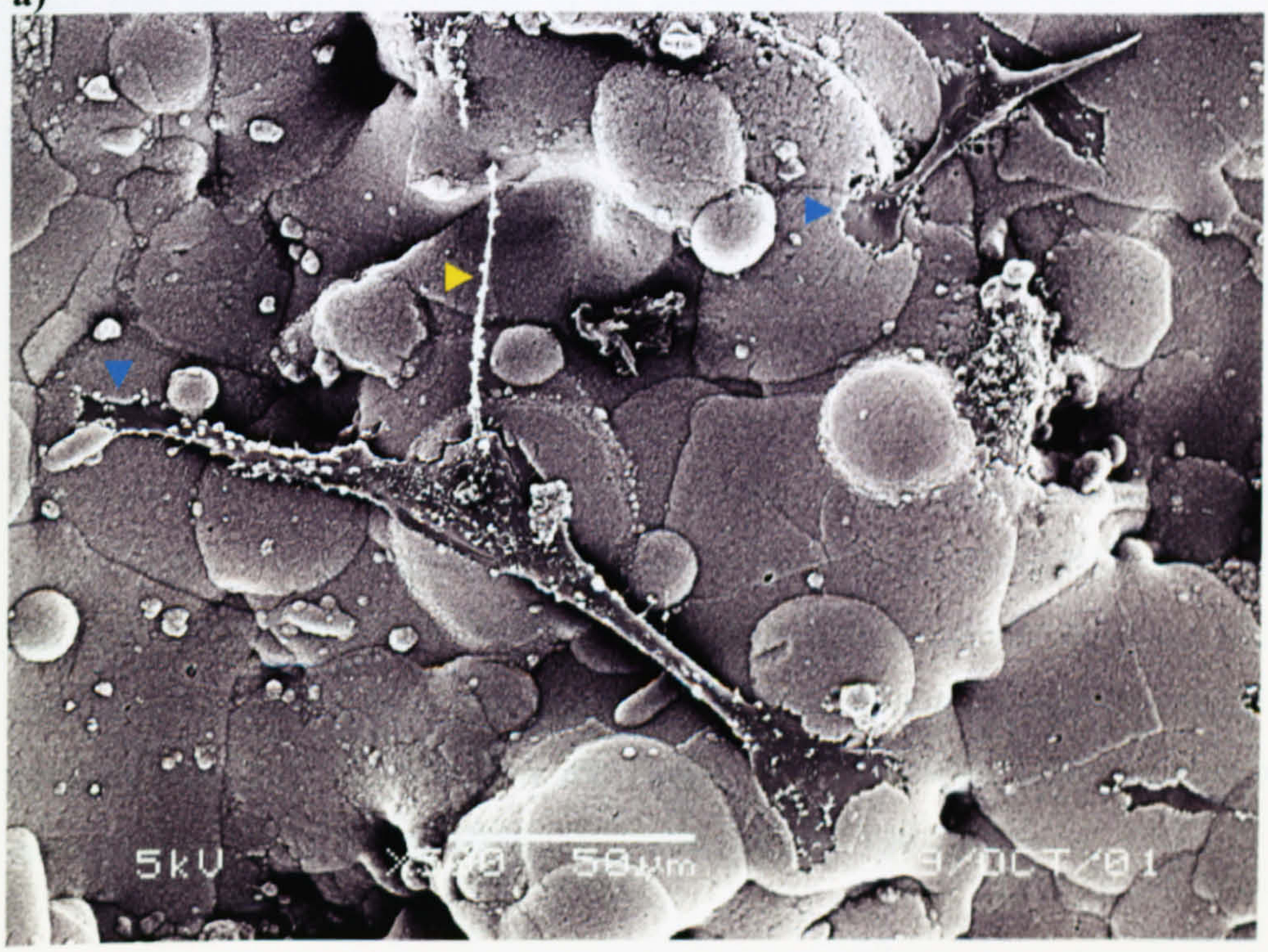
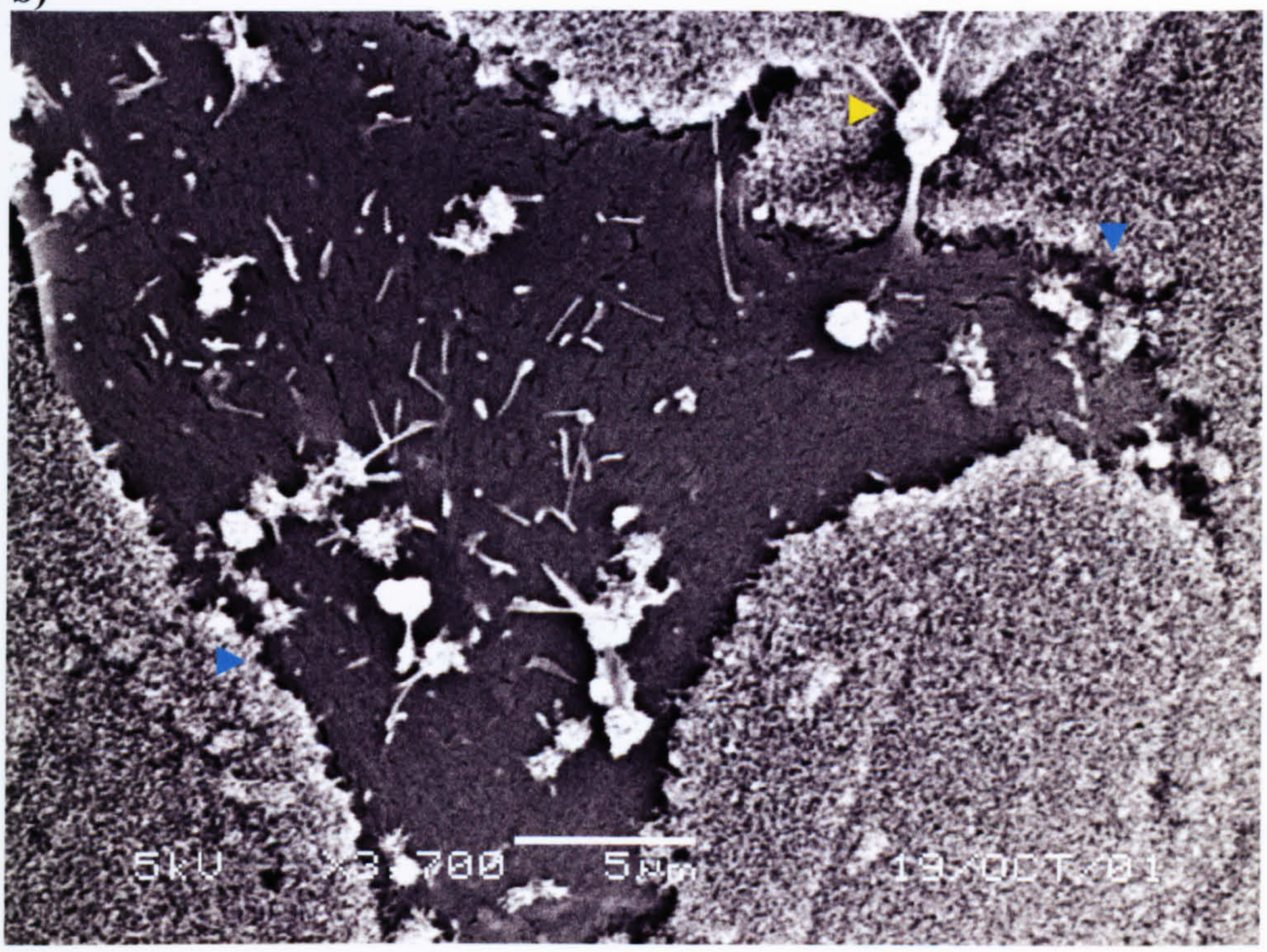


Figure 2. 8: MSCs cultured on HA discs in standard medium, note the embedding of the cells into the HA (blue arrows) after a) 1 day bar = 50µm & b) 7 days bar = 5µm. Yellow arrows indicate cell attachment processes.

a)



b)



3.3.1.3 Protein assays of osteoblastic differentiation

3.3.1.3.1 ALP production

ALP was measured as a marker of the presence of active osteoblasts in culture at time intervals during a 30-culture period. As the cells may not have increased in number at the same rate, the total DNA content of the samples was measured and used to standardise protein results, as in Chapter 1. The results were found not to follow a normal distribution on Kolmogorov-Smirnov and Shapiro-Wilk testing, thus non-parametric statistical tests were applied in the form of a Mann Whitney U test.

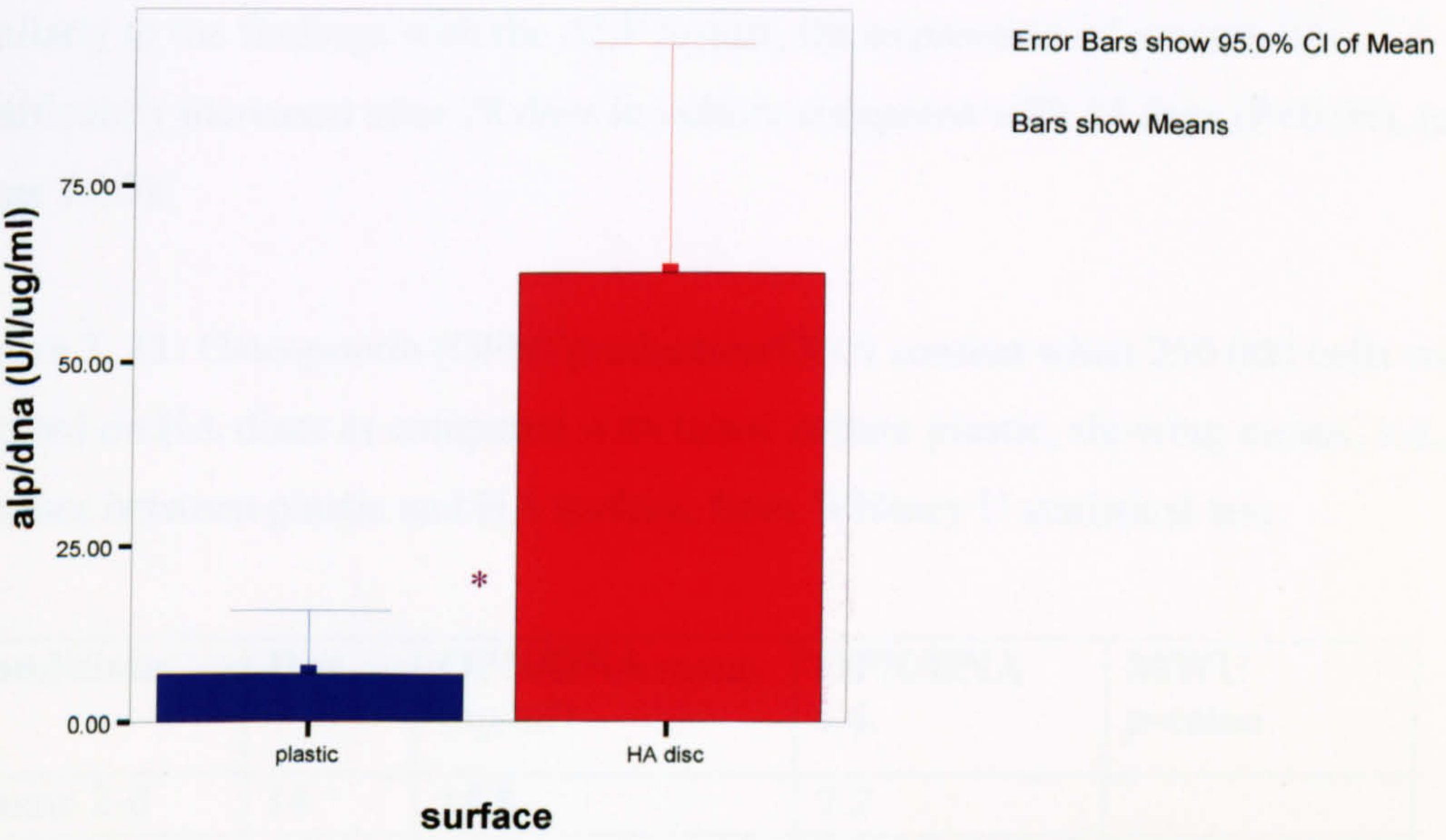
When 25,000 cells were cultured on HA surface as compared to a tissue culture plastic control there was no significant difference in the amount of ALP/DNA produced by the cells over 30 days ($P>0.05$). Therefore, the experiment was repeated with a higher cell density, 250,000 cells per sample. When the cell number was increased the production of ALP/DNA on HA was found to be significantly greater than on the control after 14 & 28 days ($P<0.005$), (see figure 2.9 & 2.10).

Figure 2. 9: ALP production and DNA content when 250,000 cells were cultured on a 2-d surface, showing the means, standard deviations (s.d.), and p-values resulting from Mann Whitney U statistical tests.

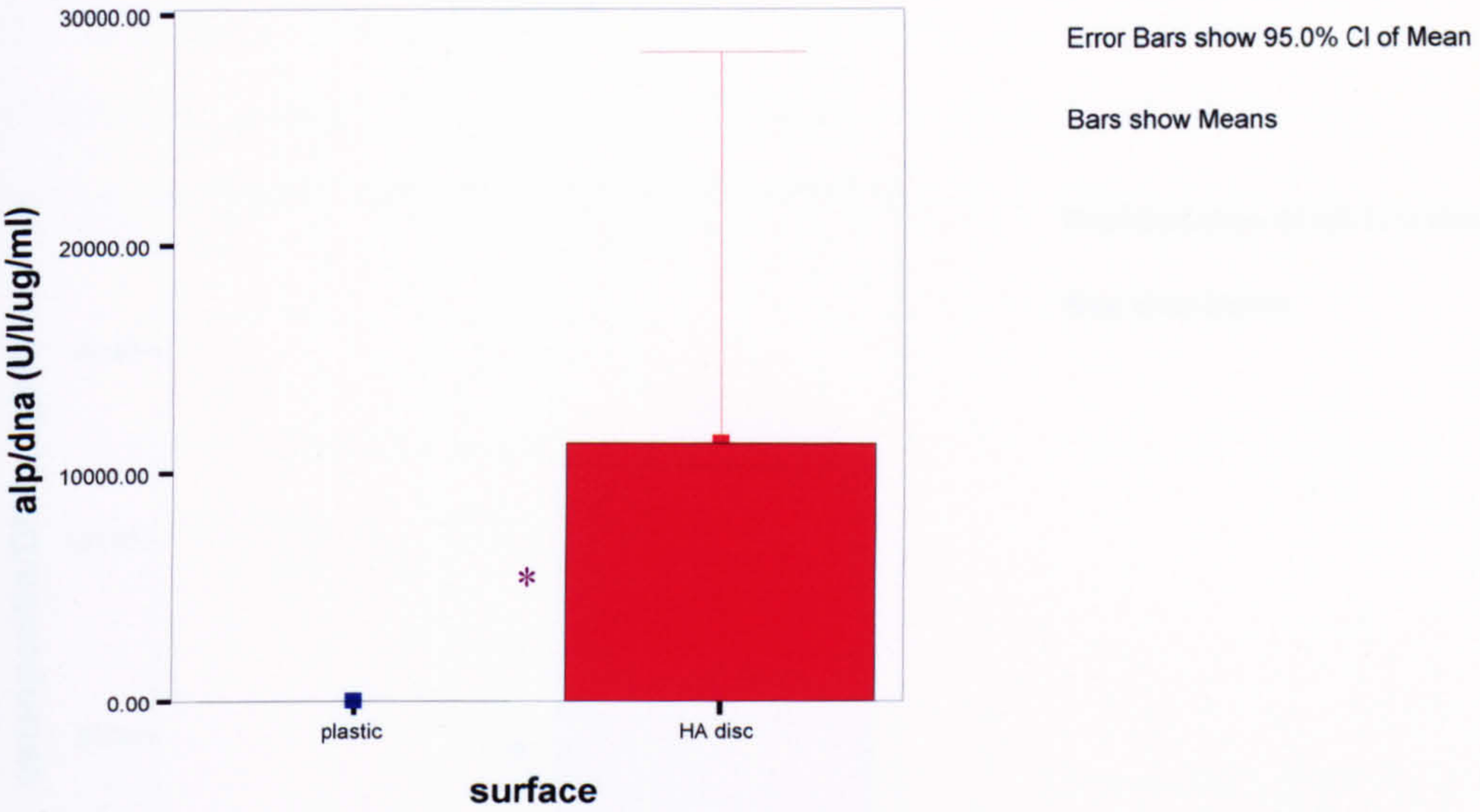
Conditions	**Day**	**ALP/DNA** mean (U/l/mg/ml)	**ALP/DNA** s.d.	**MWU** p-value
Plastic 2-d	14	10.4	9.7	
HA 2-d	14	60.5	26.5	0.001
Plastic 2-d	28	25.7	8.7	
HA 2-d	28	10549.5	9858.2	0.001

Figure 2. 10: Bar charts showing the production of ALP/DNA for MSCs grown on the plastic control (blue) compared with HA discs (red) after a) 14 days and b) 28 days, *P<0.005.

a)



b)



3.3.1.3.2 Osteopontin production

The quantity of osteopontin protein was measured after 14 days in culture comparing HA discs with the control. The production of osteopontin/DNA by MSCs was significantly greater for cultures on HA than on plastic ($P<0.005$), (see figure 2.11 & 2.12).

Similarly to the findings with the ALP results, the expression of osteopontin significantly increased after 28 days in culture compared with 14 days ($P<0.05$), (see figure 2.13).

Figure 2. 11: Osteopontin (OPN) production/DNA content when 250 000 cells were cultured on HA discs as compared with tissue culture plastic, showing means, s.d., and p-values between plastic and HA surface, from Whitney U statistical test.

Conditions	**Day**	**OPN/DNA** mean (ng/ml)	**OPN/DNA** s.d.	**MWU** p-value
Plastic 2-d	14	14.8	7.2	
HA 2-d	14	119.2	59.9	0.004
HA 2-d	28	4312.0	5781.0	0.01

Figure 2. 12: Bar charts showing the production of osteopontin/DNA for MSCs grown on the plastic control (blue) compared with HA discs (red) after 14 days, *P<0.005.

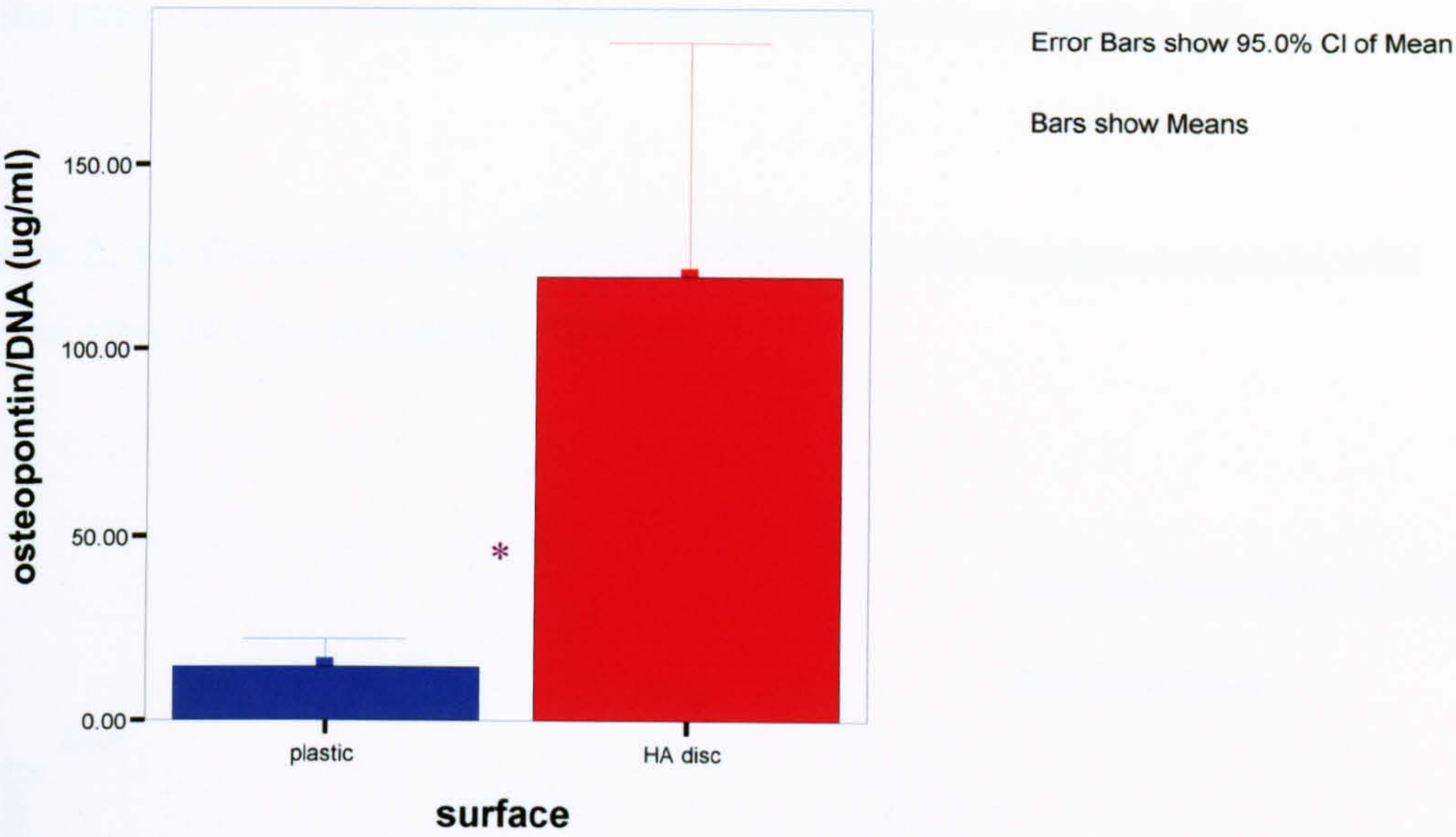
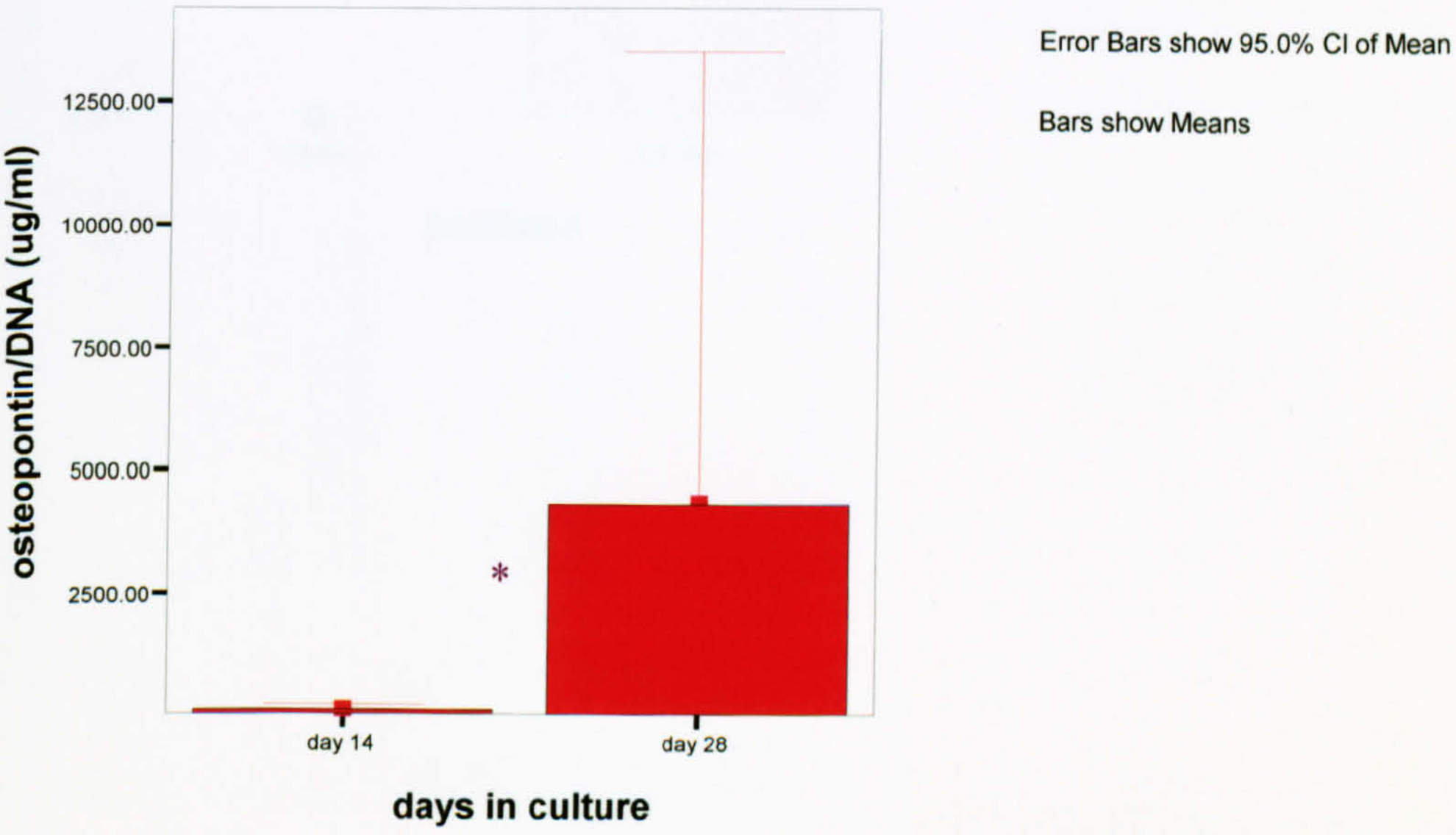


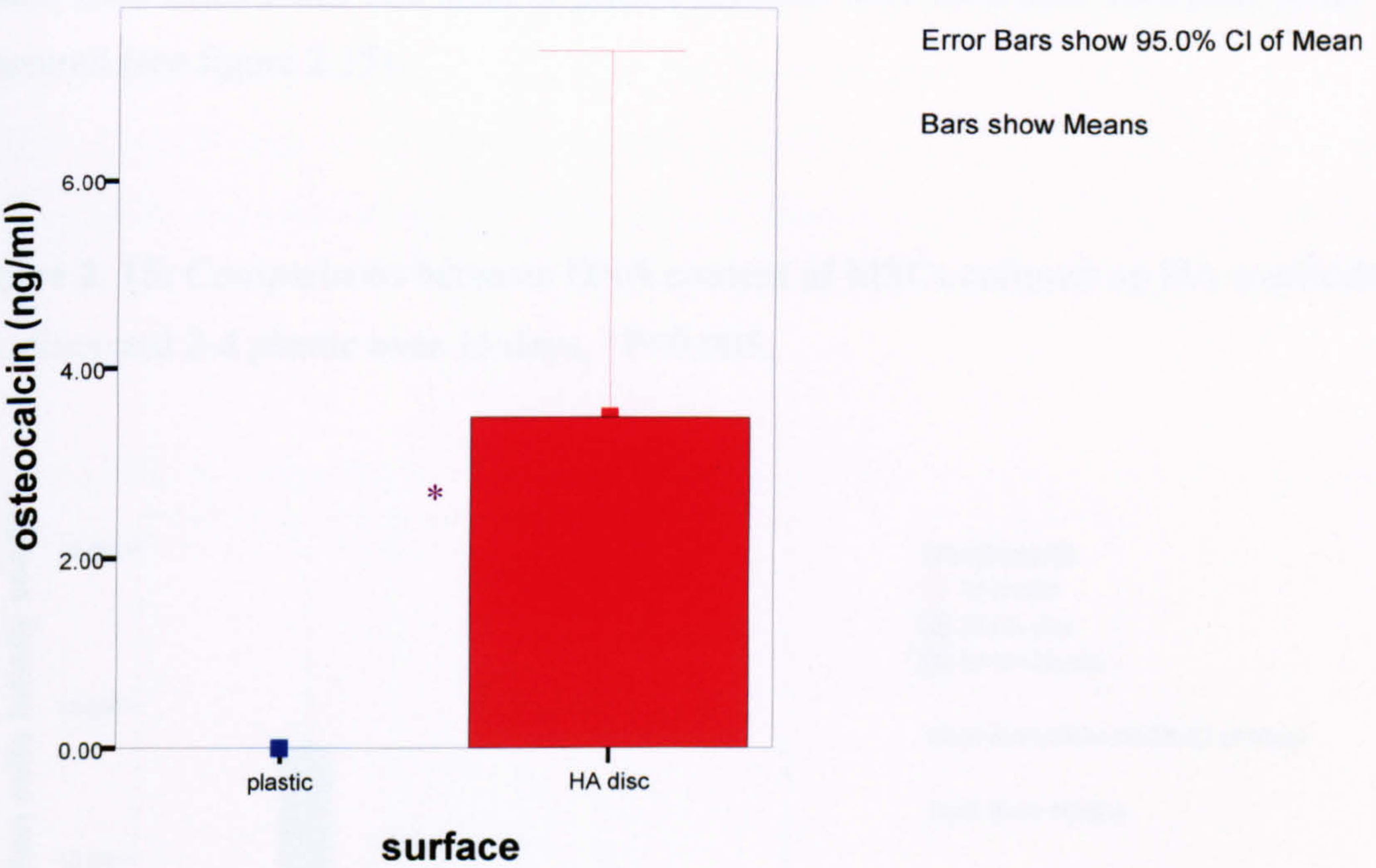
Figure 2. 13: Bar charts showing the production of osteopontin/DNA for MSCs grown on HA discs (red) after 14 days compared with 28 days, *P<0.05.



3.3.1.3.3 Osteocalcin production

Osteocalcin production was measured as a further marker of osteoblastic extracellular matrix production. Osteocalcin/DNA was produced when the higher density of MSCs (250,000) was cultured for 14 days on HA discs, but cells at the same higher density on the plastic control did not produce any osteocalcin (see figure 2.14).

Figure 2. 14: Osteocalcin production by MSCs on a HA surface compared with control after 14 days in culture, *P<0.05.



3.3.2 MSCs on 3-Dimensional Porous Hydroxyapatite Scaffold

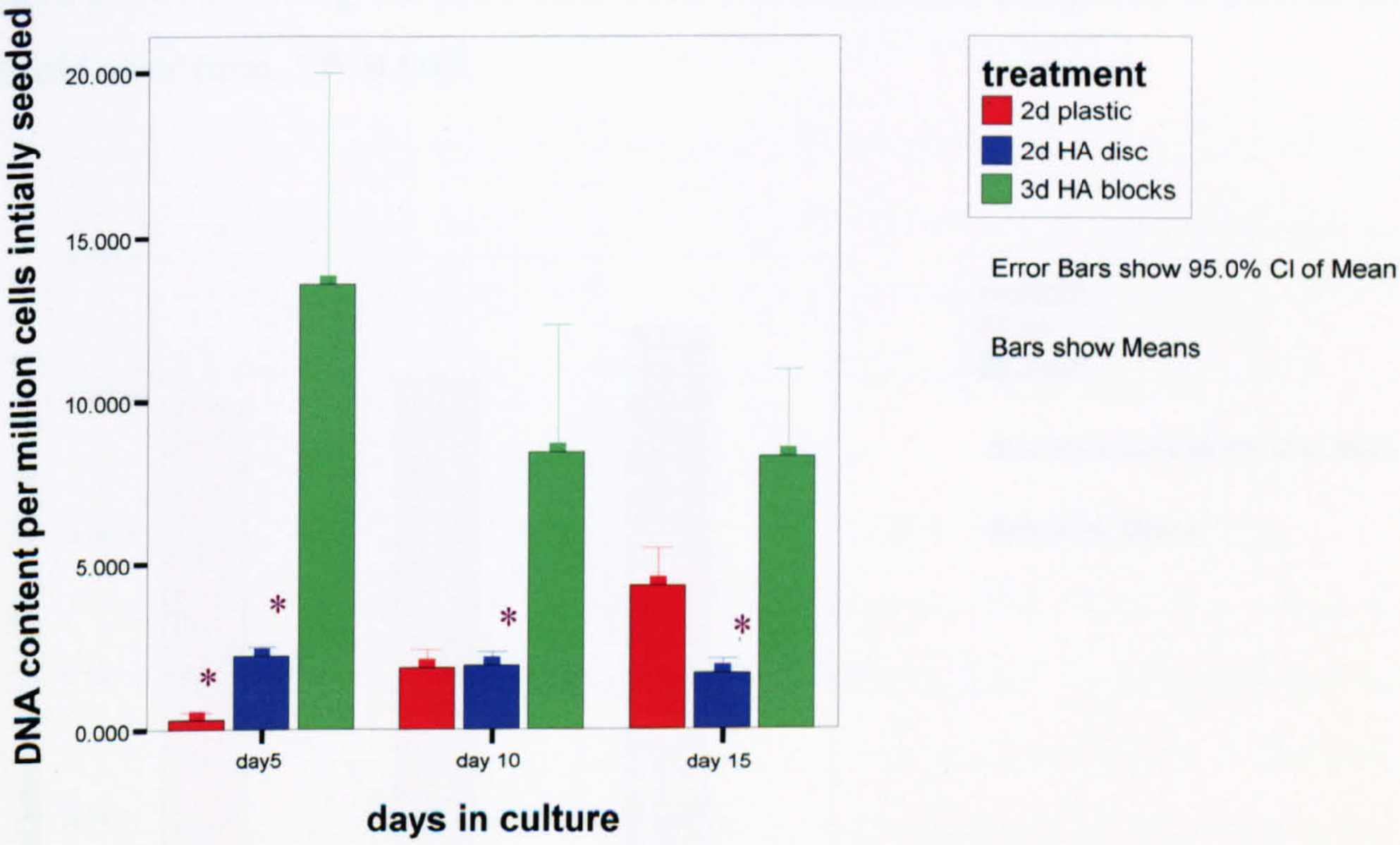
3.3.2.1 Assessment of proliferation rate on scaffolds

3.3.2.1.1 DNA level results

The DNA content of MSCs cultured on HA scaffolds did not significantly increase over the 15-day culture period ($P>0.05$). This is similar to DNA results for MSCs cultured on 2-d HA see results above (figure 2.1).

Following standardisation for the initial number of cells seeded on each of the substrates, the DNA content of the cells grown on HA scaffolds was significantly greater than when either HA discs or plastic controls were used after each time point measured (see figure 2.15).

Figure 2. 15: Comparisons between DNA content of MSCs cultured on HA scaffolds, HA discs and 2-d plastic over 15 days, *$P<0.005$.



3.3.2.1.2 Alamar blue assay results

Alamar blue absorbance was significantly greater when MSCs were cultured on the HA scaffold compared to the plastic control over 10 days ($P<0.005$). However, the Alamar blue results were very low over the first 10 days in culture on the plastic scaffold and by 15 days no absorbance could be detected (see figure 2.16).

The Alamar blue absorbance did not increase significantly over time for the MSCs cultured on HA scaffolds (see figure 2.17), similarly to the MSCs on HA discs (see results above, figure 2.2).

The Alamar blue absorbance from MSCs cultured on the HA scaffold was compared with the results from the HA discs, following standardization for initial cell seeded density. The absorbance was shown to be significantly greater for the MSCs grown on HA scaffolds compared to the HA discs after 10 days in culture ($P<0.005$), (see figure 2.17).

Figure 2. 16: Showing the proliferation rate of MSCs HA compared to porous plastic scaffold over time, *$P<0.005$.

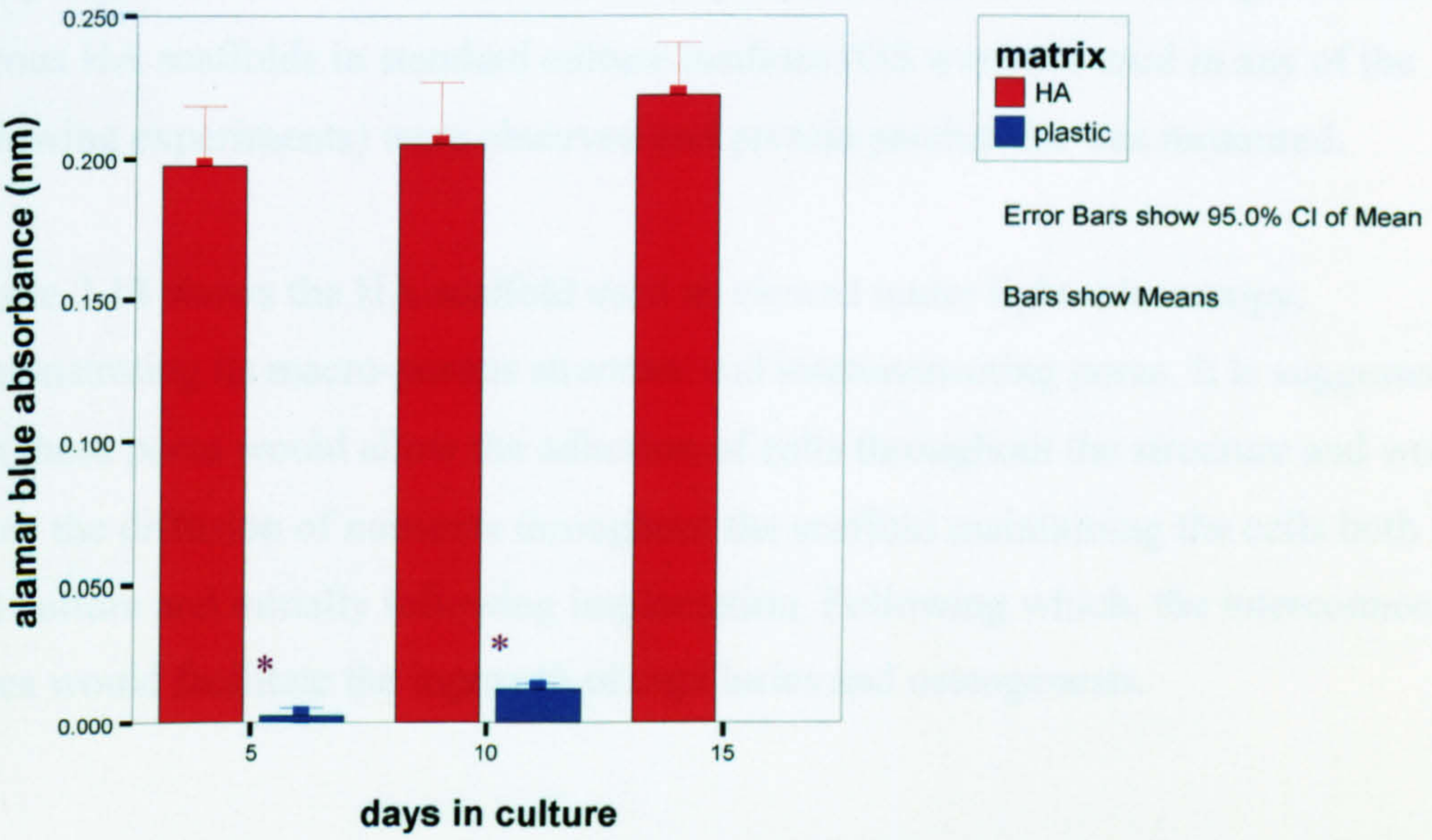
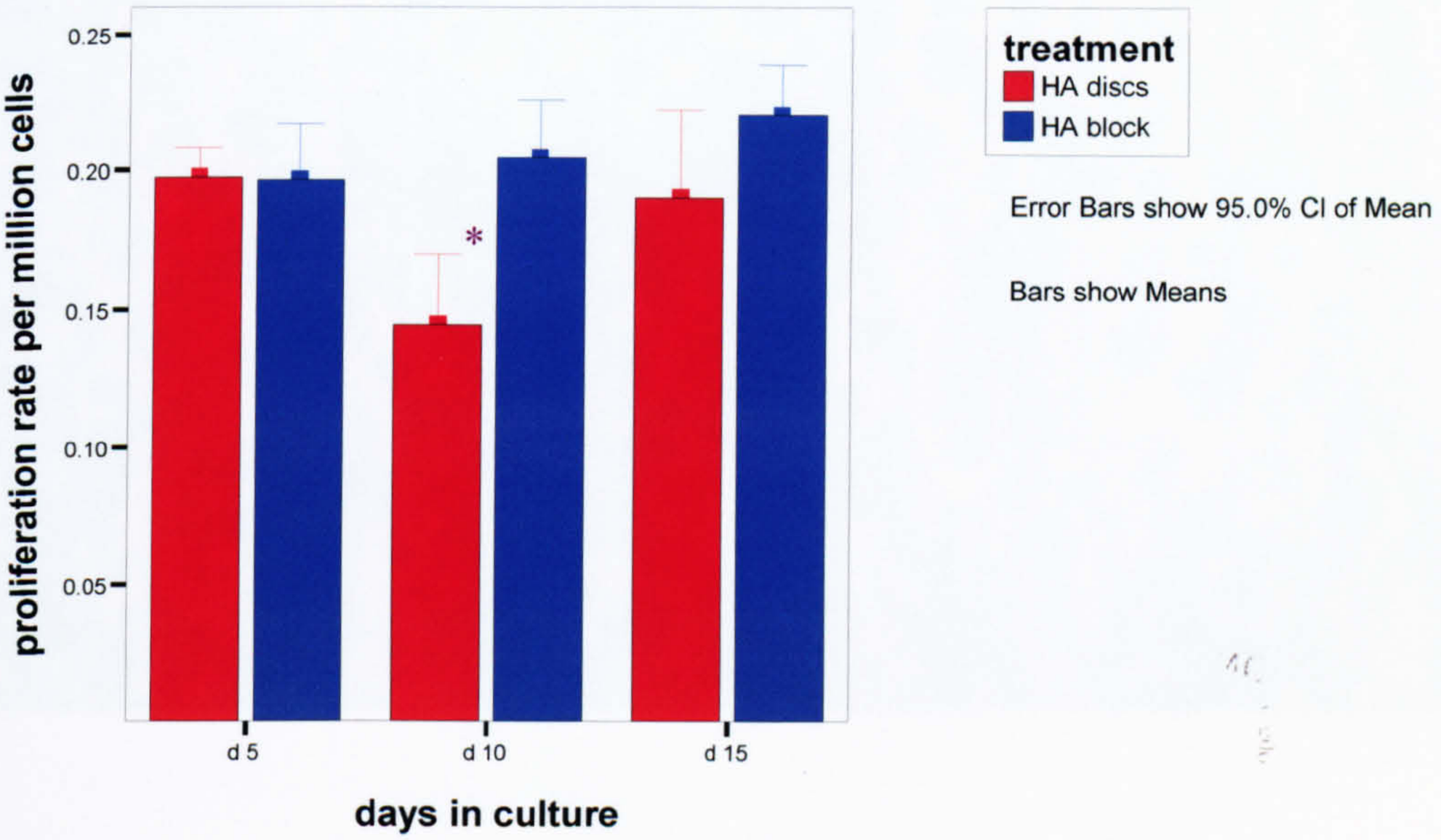


Figure 2. 17: Comparison between the proliferation rate of MSCs on porous HA scaffolds and HA coated discs over the 15-day culture period, by calculating Alamar blue absorbance per million cells seeded, *P<0.005.

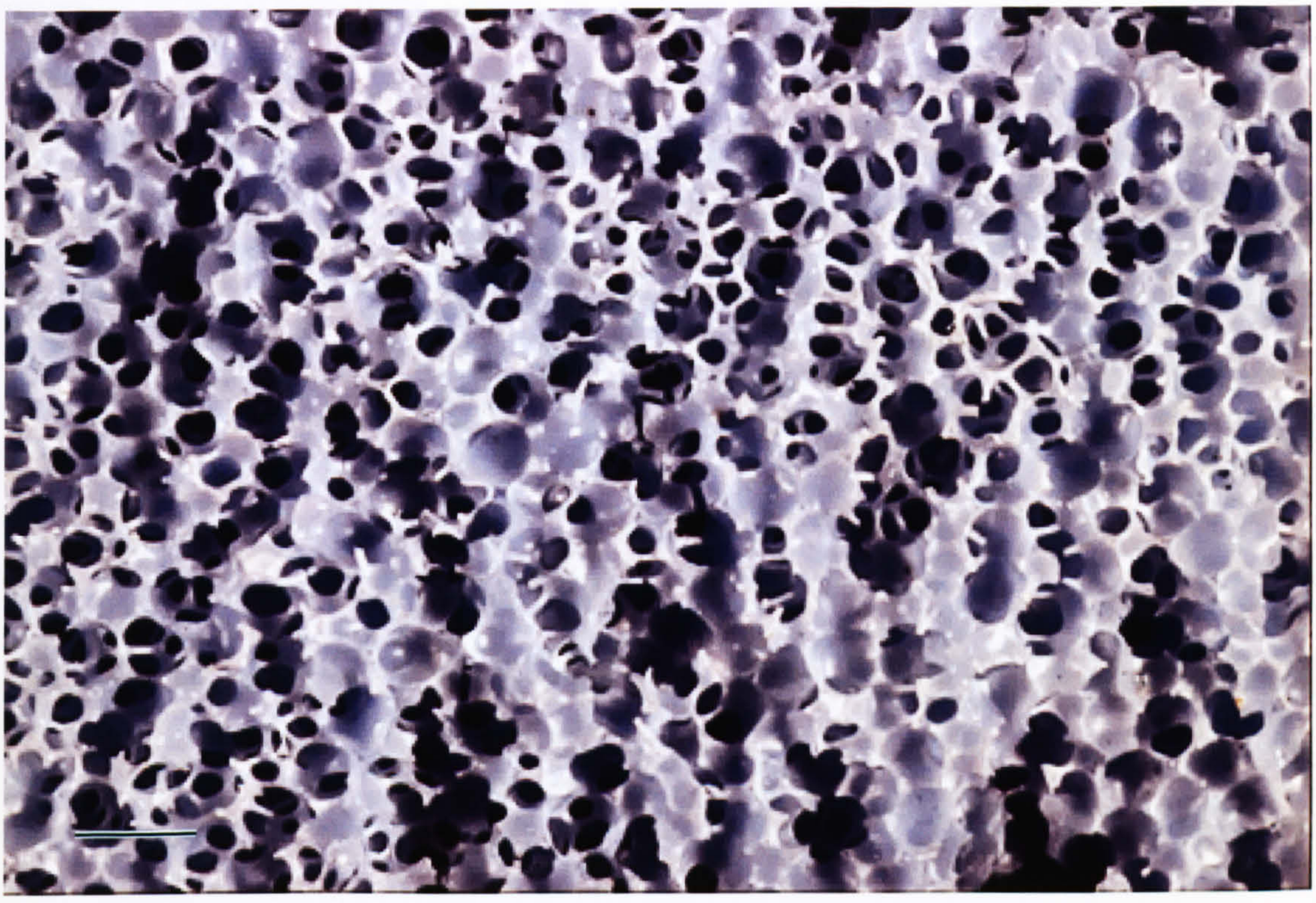


3.3.2.2 Observational studies of MSCs grown on HA scaffolds

In the first part of this chapter the response of MSCs cultured on 2-d HA surface was compared to tissue culture control. In this part, the behaviour of MSCs grown on porous HA scaffolds in standard culture medium (OS were not used in any of the following experiments) were observed and protein production was measured.

Figure 2.18 shows the HA scaffold used as viewed under light microscopy, demonstrating its macro-porous structure and interconnecting pores. It is suggested that these pores would allow the adhesion of cells throughout the structure and would allow the diffusion of nutrients throughout the scaffold maintaining the cells both in cell culture and initially following implantation. Following which, the interconnecting pores would facilitate the ingrowth of capillaries and osteogenesis.

Figure 2. 18: A picture of the porous HA scaffold, bar = 1000μm.



3.3.2.2.1 Light microscopy observations

The porous HA scaffolds were stained with toluidine blue after 5 days in culture, showing that MSCs were adhering and growing on this culture surface (see figure 2.19). At higher magnification the individual cells could be observed and after 5 days the fibroblastic shape could be determined (see figure 2.20).

A porous plastic scaffold with the same structure as the porous HA was used as a control. However, when the plastic scaffold control was stained with toluidine blue after a similar time period cells could not be seen attaching to the surface, only evidence of cell debris. As a consequence of this result and the Alamar blue readings it was felt that the plastic scaffold was not a reliable substrate for cell growth. Therefore, for the remaining experiments the results for MSCs cultured on 3-d HA scaffolds were compared with 2-d HA, which acted as a control for the 3-d structure.

Figure 2. 19: After staining with toluidine blue, cells were observed adhered to all surfaces of the HA scaffold, as seen by the darker areas in this photograph after 5 days in culture.

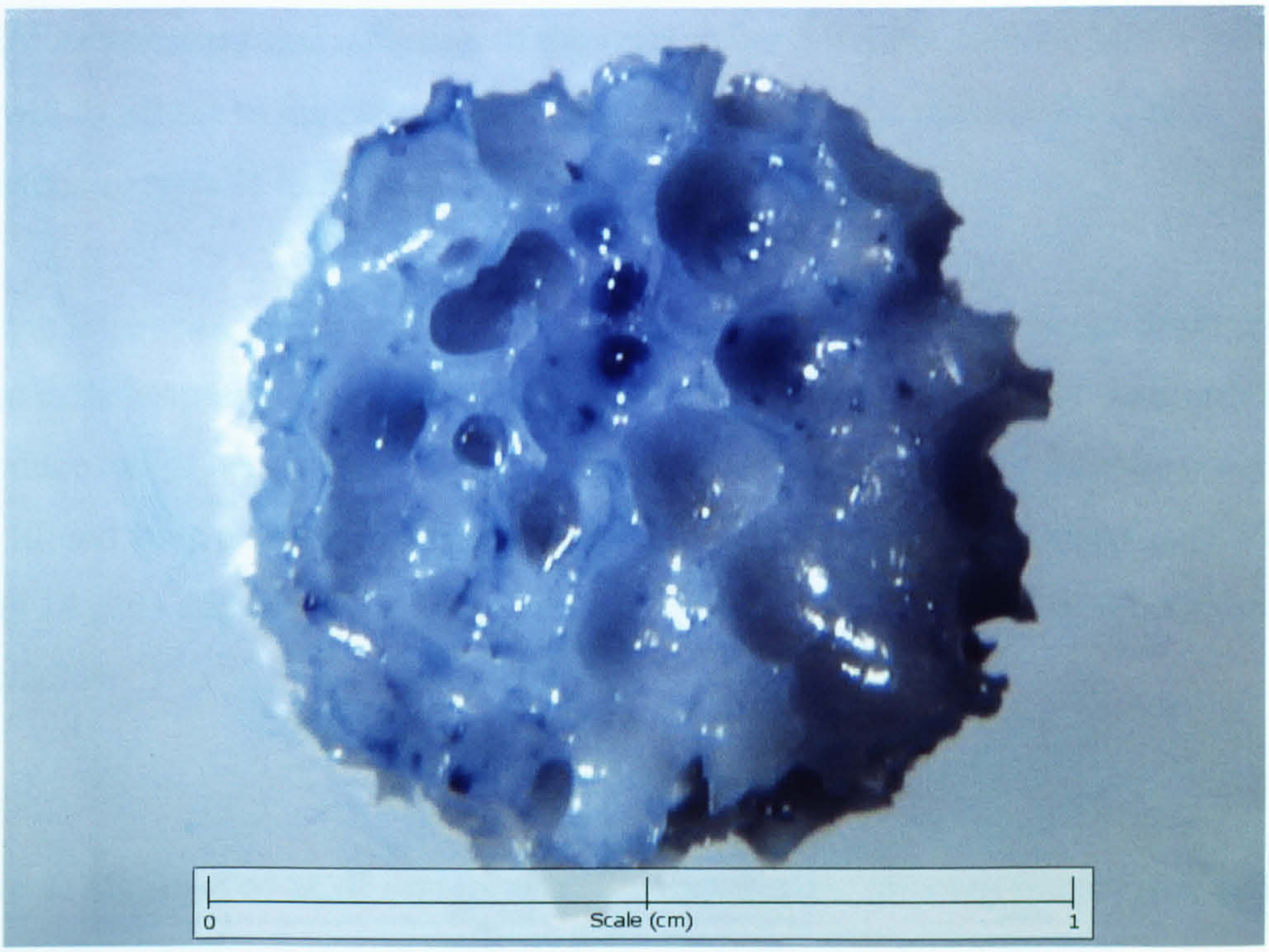
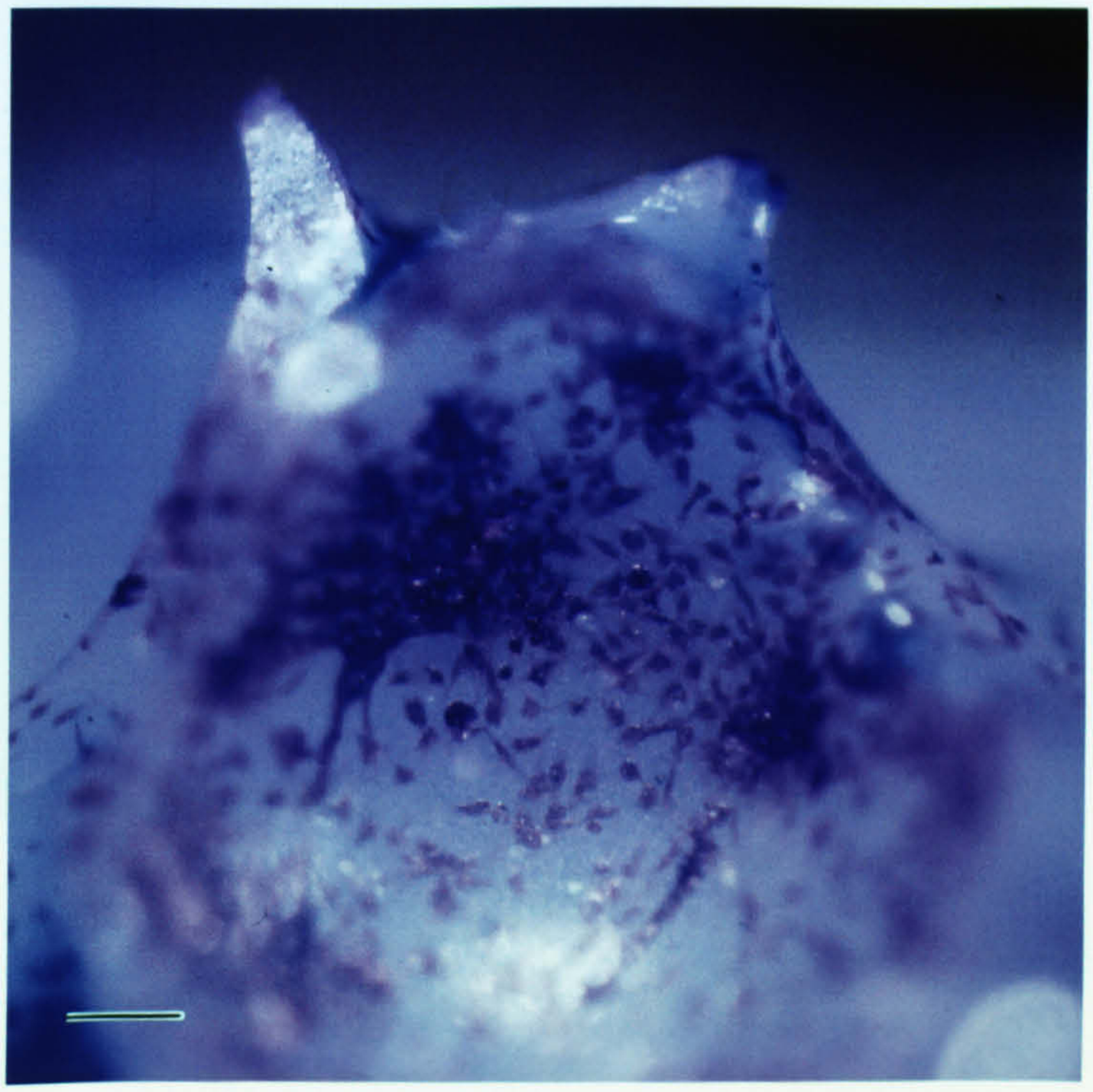


Figure 2. 20: Fibroblastic-shaped cells characteristic of MSCs seen on part on porous HA scaffold after staining with toluidine blue after 5 days in culture, bar = 250μm.



3.3.2.2.2 SEM results

The MSCs were observed at a higher resolution under SEM, showing the surface of the HA, morphology and adhesion of the cells. After 5 days in culture, MSCs were observed to adhere to the HA scaffold, forming clusters and maintained their characteristic spindle-like shape (see figure 2.21).

Over the following 15-day culture period the MSCs were noted to change from spindle shaped cells becoming more brick-like (see figures 2.22 & 2.23). Cells were observed to produce multiple processes attaching them to the HA surface and neighbouring cells. Round cells were also seen characteristic of active osteoblasts (see figure 2.22). By day 14 cells had covered the HA surface forming a carpet of adherent cells (see figure 2.23).

Figure 2. 21: SEM of MSC on HA scaffold after 5 days, showing fibroblastic cells (yellow arrows) spread over the HA surface, magnification bar = 200μm.

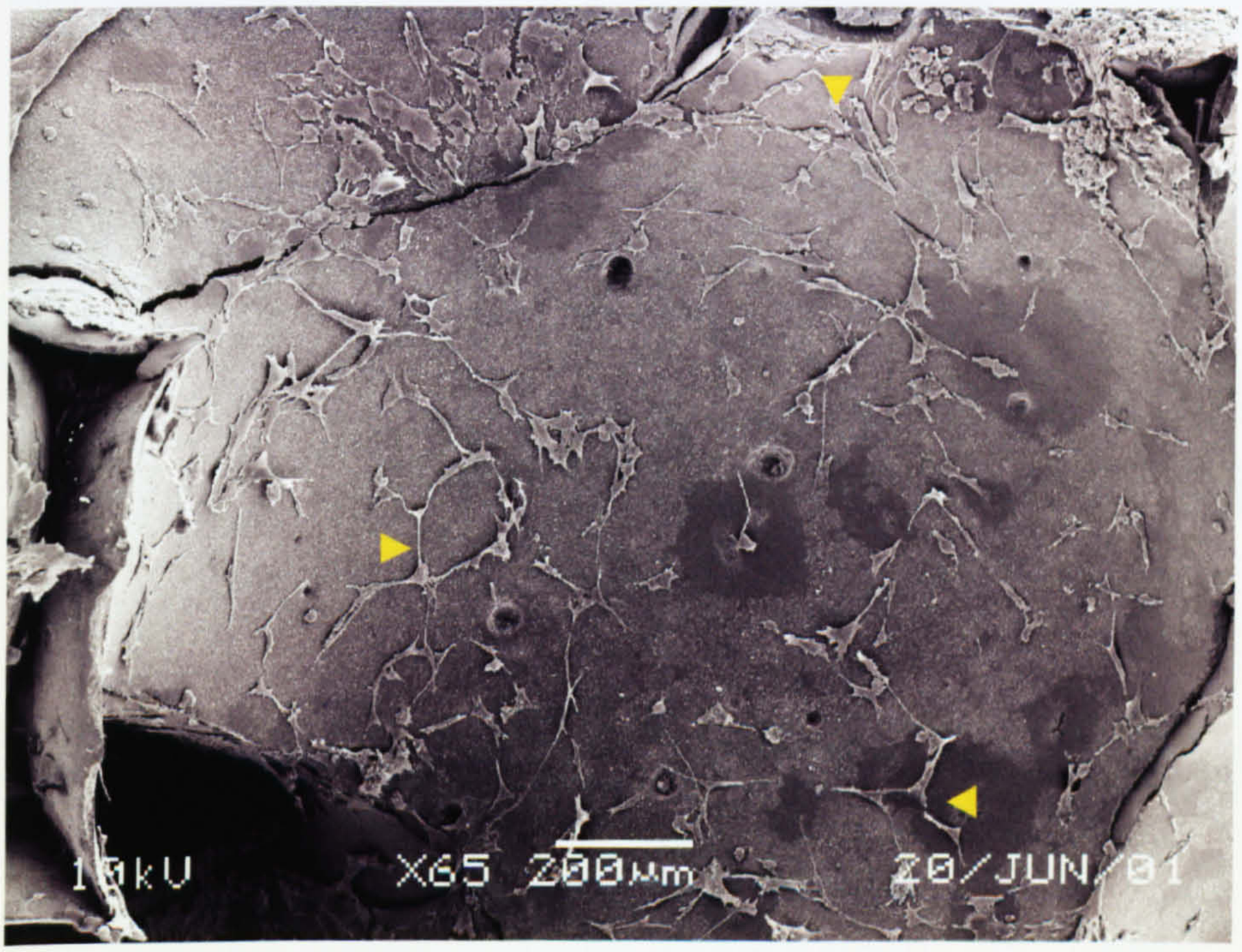
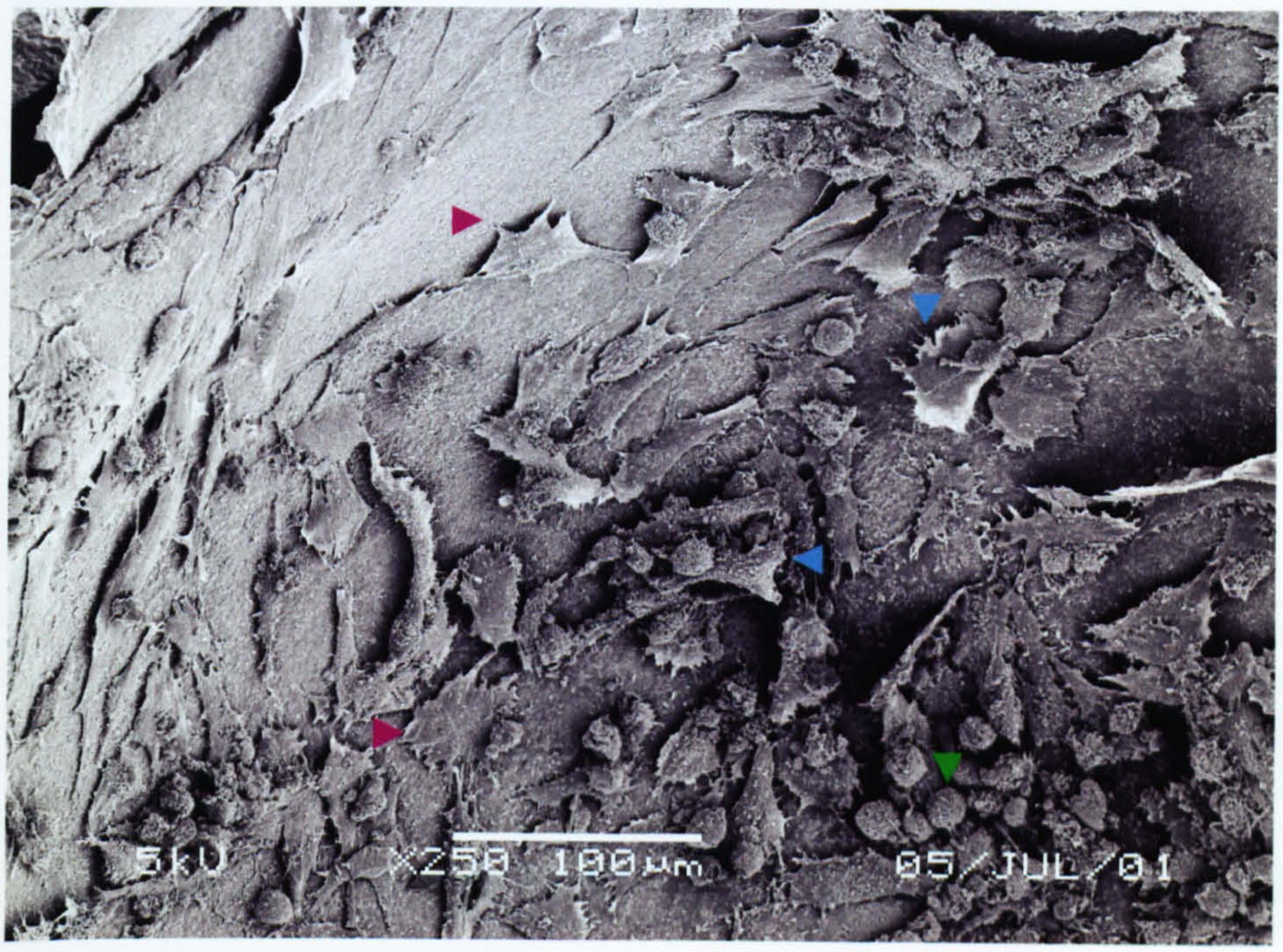


Figure 2. 22: SEM of MSCs on porous HA after 7 days, a) showing brick-shaped cells (blue arrows), round cells (green arrows) and cell processes attaching them to HA (pink arrows), bar = 100μm, b) at higher magnification, arrows indicating cells as before and bar = 50 μm.

a)



b)

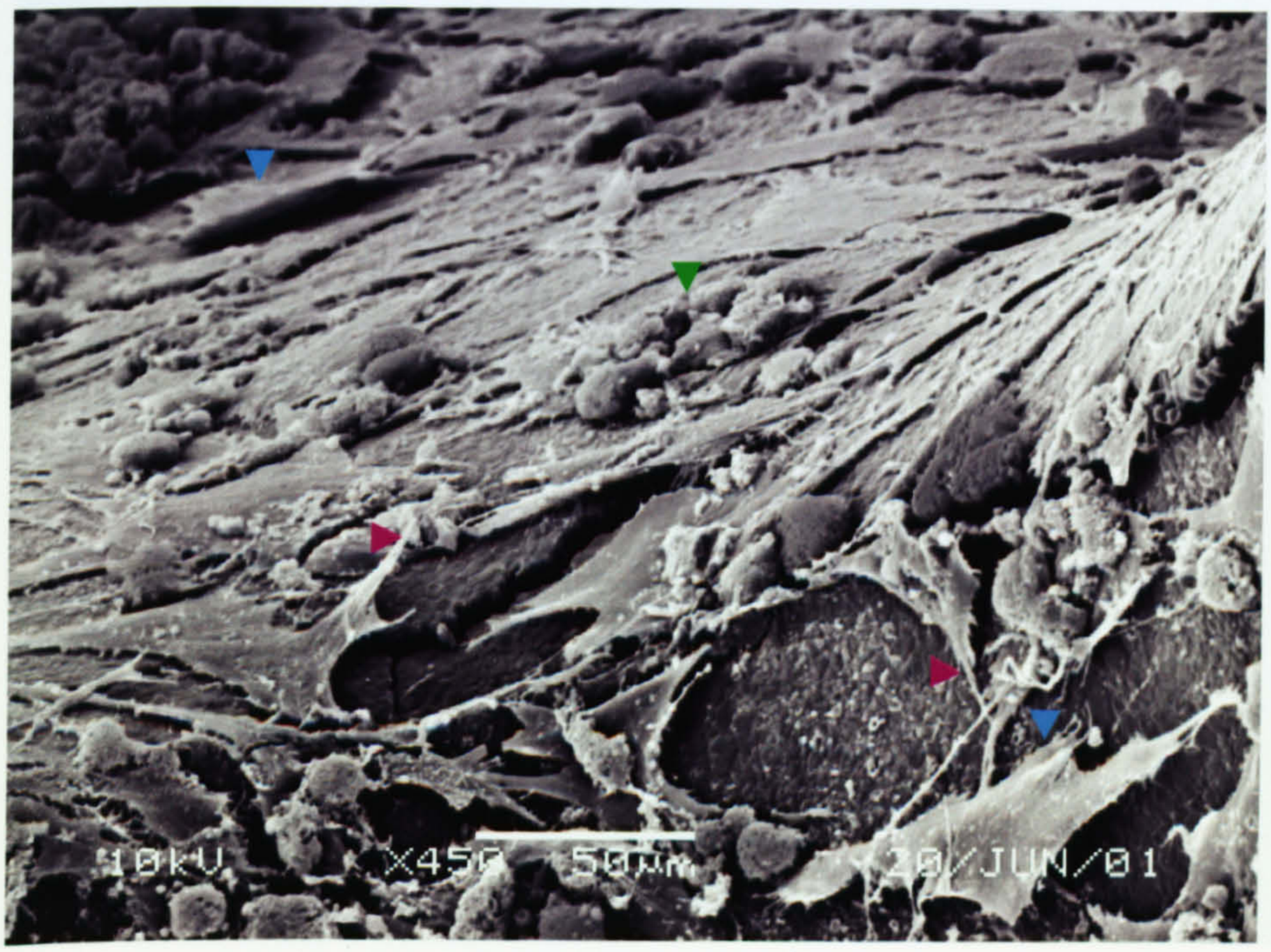
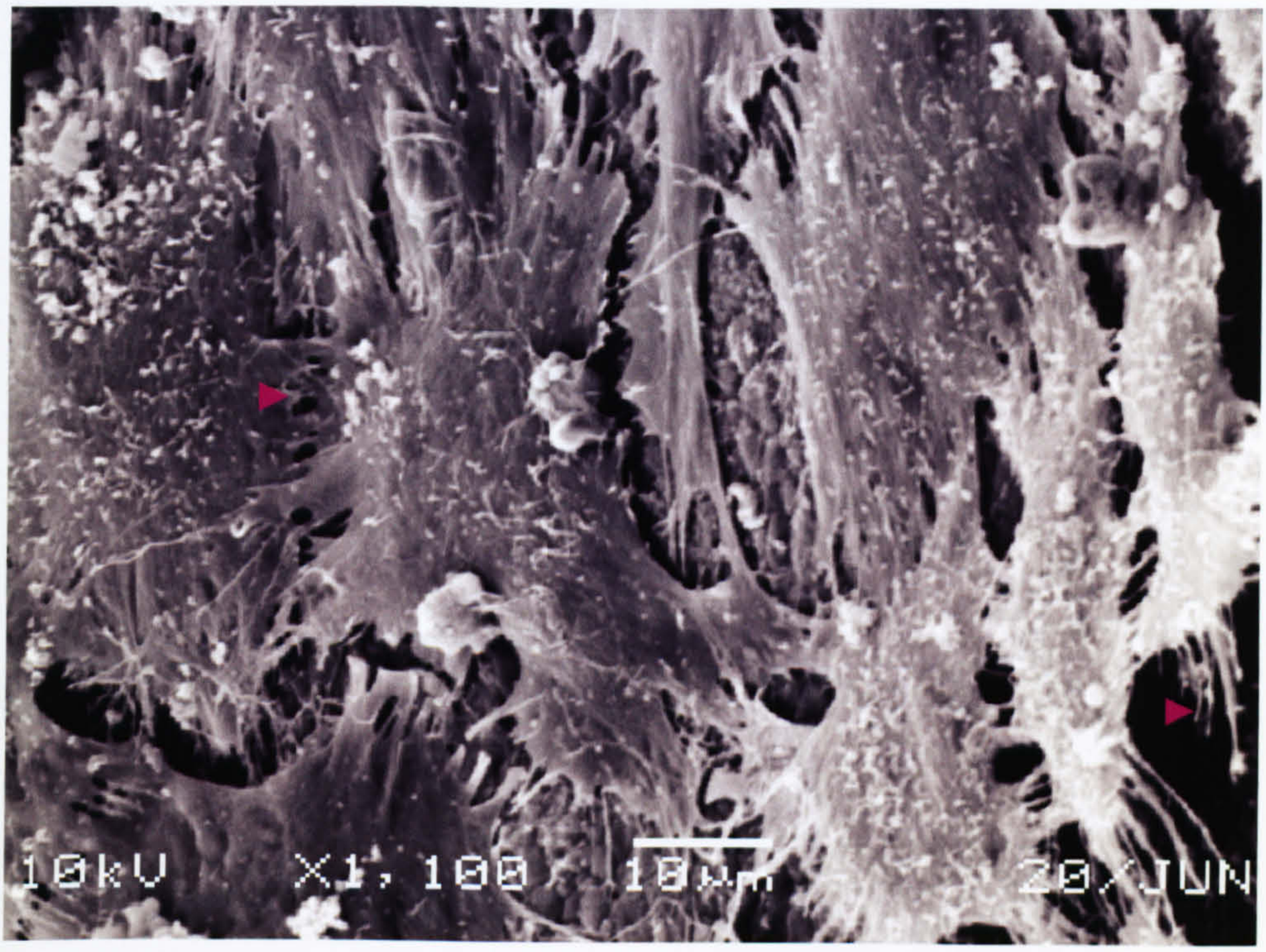


Figure 2. 23: SEM of MSCs cultured on porous HA for 14 days, a) showing cells with multiple processes (pink arrows), bar = 10μm, b) cells covering the HA surface, bar = 10μm.

a)



b)

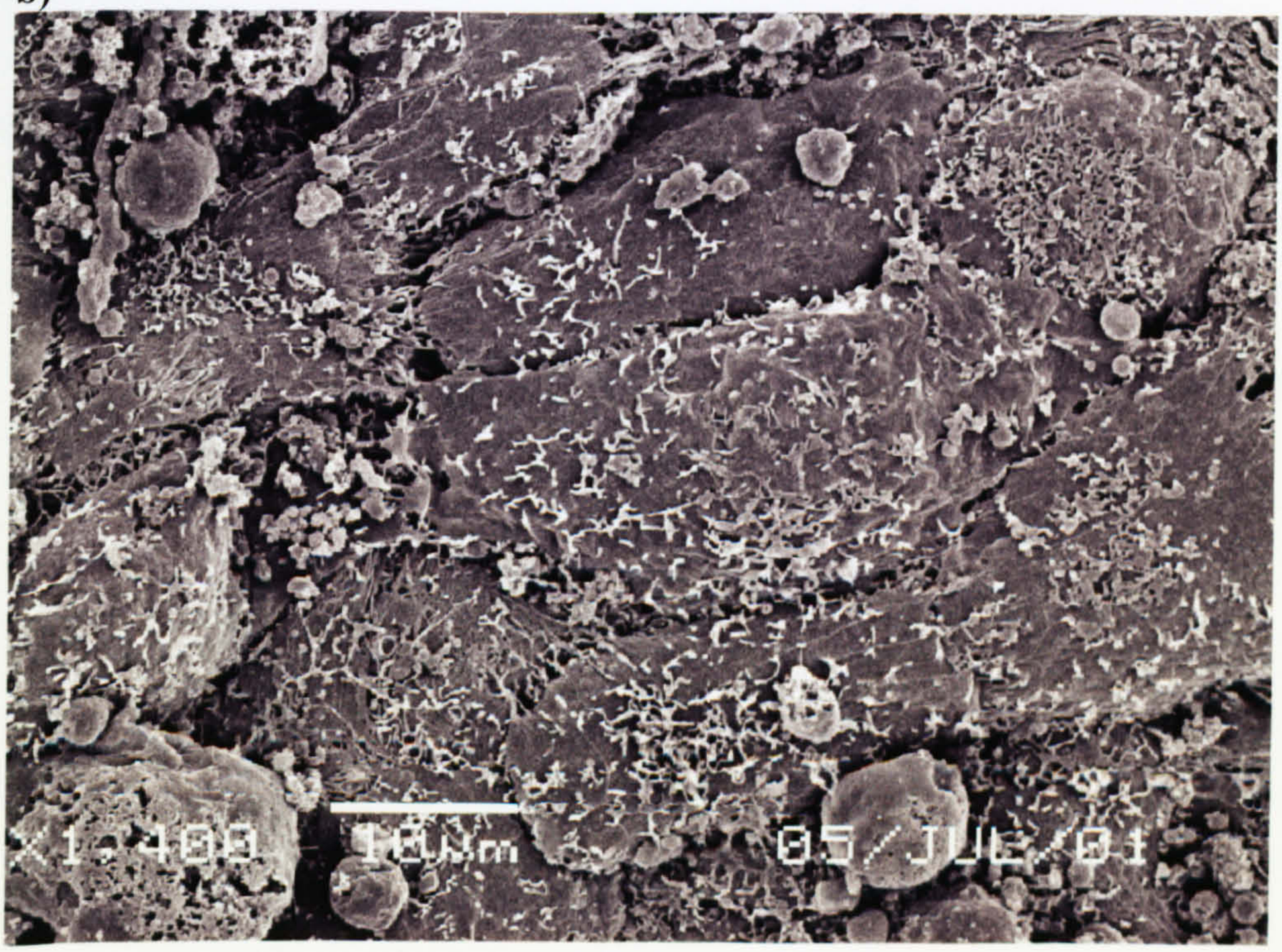


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Figure 2. 24: Cells containing nucleus (N) attaching to the edge of HA scaffold (E) by cell processes (P) after 28 days in culture. Bar = 0.5μm.

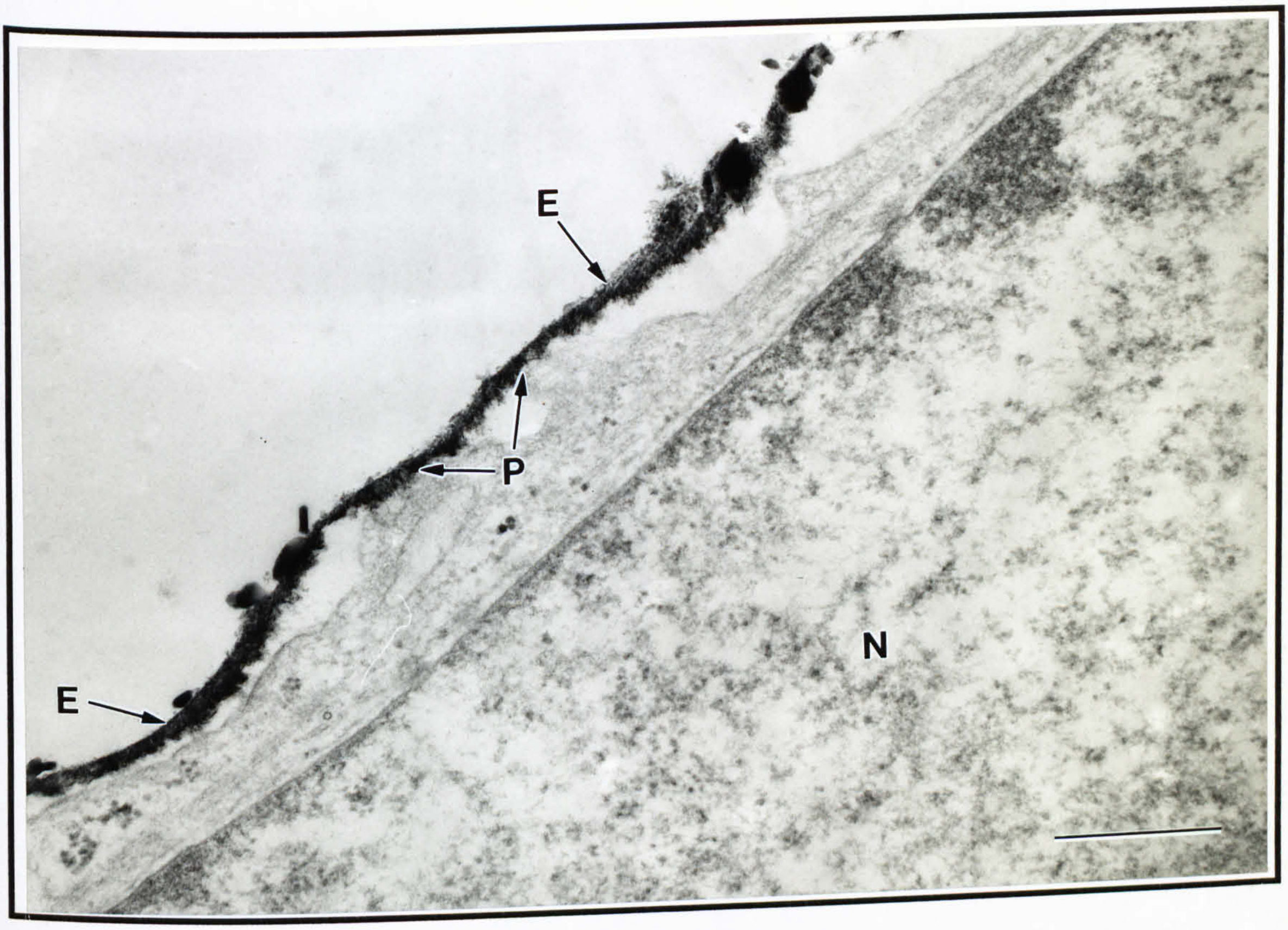


Figure 2.24

Figure 2. 25: Cells cultured on HA scaffold for 28 days, showing cell processes (large arrows) and containing nuclei (N), mitochondria (small arrows), endoplasmic reticulum (arrowheads) and glycogen (G). There is also evidence of collagen fibrils (C) between cells. Bar = 2μm.

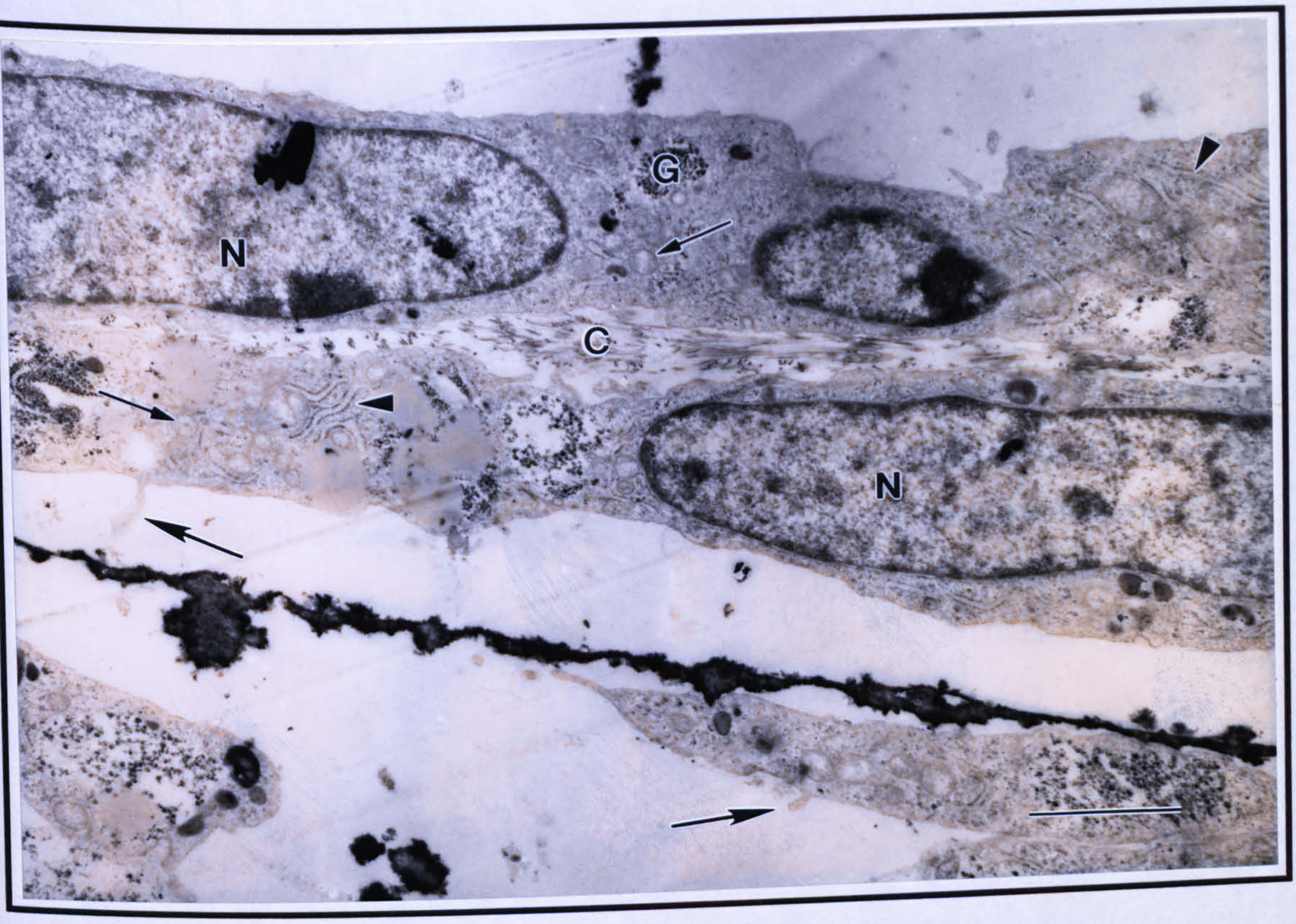


Figure 2.25

3.3.2.4 Protein assays of osteoblastic differentiation on HA scaffold

3.3.2.4.1 ALP Results

Protein assay levels were divided by total DNA to standardise the results for each sample, as for HA discs in the first part of this chapter. The results did not follow a normal distribution so non-parametric statistical tests were used to compare the conditions.

The production of ALP/DNA by MSCs grown HA scaffolds increased significantly over the 15-day culture period ($P<0.05$), (see figure 2.26).

The relative expression of ALP/DNA between cells cultured on HA scaffolds and HA discs. Cells cultured on HA discs expressed a greater amount of ALP than cells on HA blocks ($P<0.005$), (see figure 2.27).

Figure 2. 26: Box plot showing that the production of ALP/DNA increases with time in culture, * ($P<0.05$).

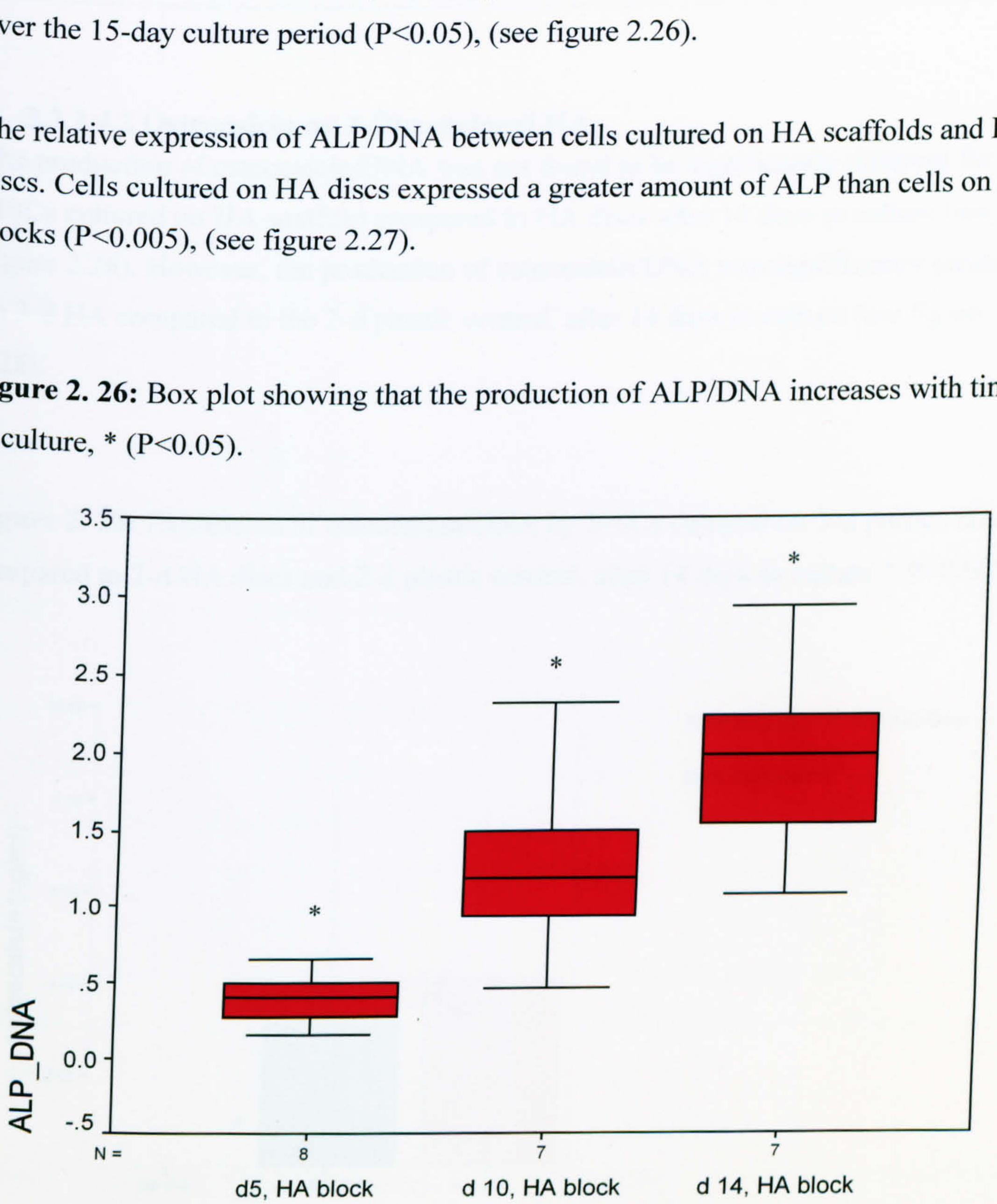


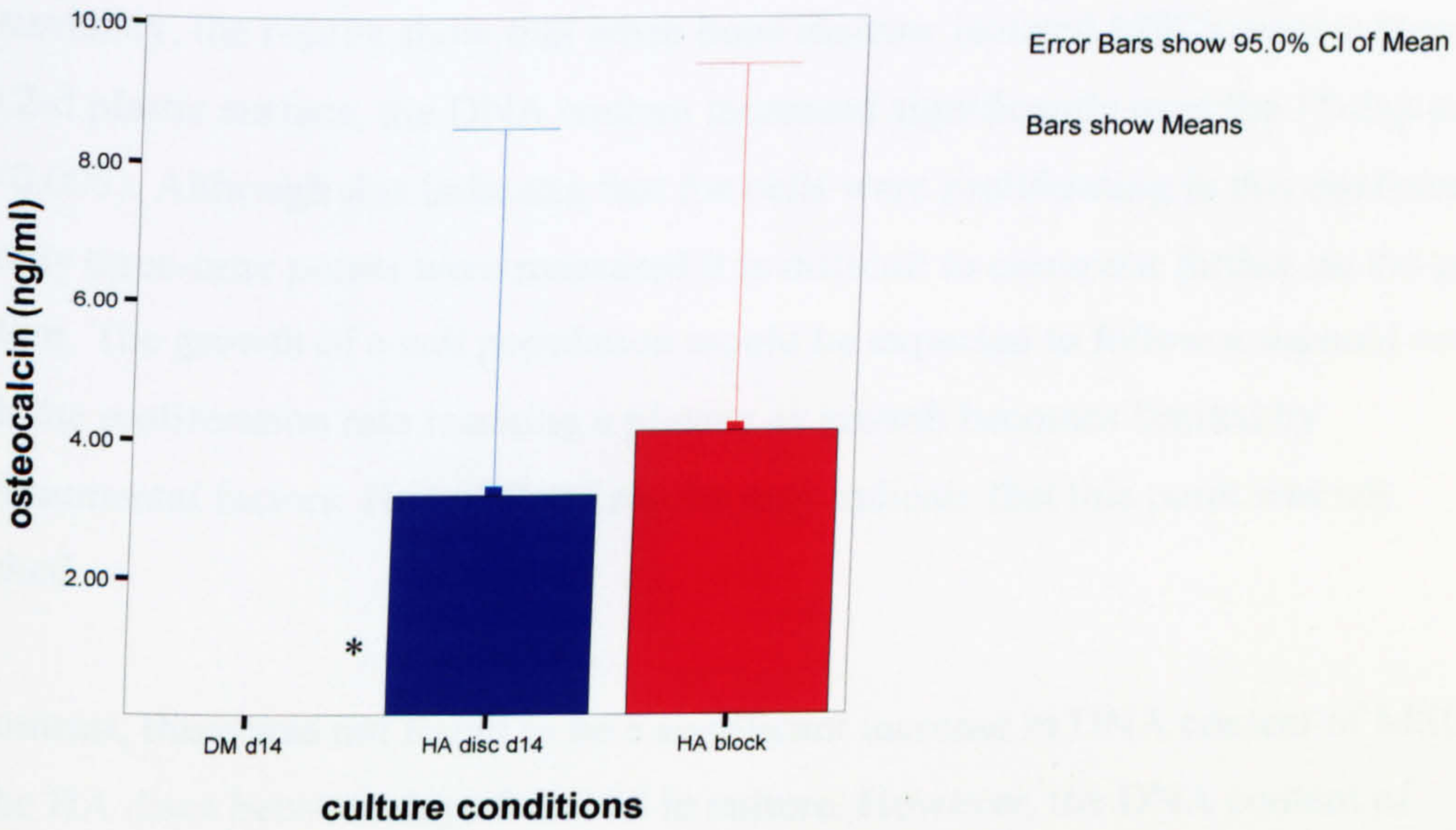
Figure 2. 27: ALP/DNA over 15 days on 2-d compared with 3-d HA

Conditions	Day	ALP/DNA (U/l/mg/ml)	ALP/DNA s.d.	MWU p-value
HA 3-d	5	0.4	1.3	
HA 2-d	5	13.9	6.4	0.002
HA 3-d	10	1.25	0.6	
HA 2-d	10	11.1	4.8	0.003
HA 3-d	15	1.9	0.7	
HA 2-d	15	16.5	7.4	0.003

3.3.2.4.2 Osteocalcin on 3-Dimensional HA

The production of osteocalcin/DNA was not found to be significantly different for MSCs cultured on HA scaffold compared to HA discs after 14 days in culture (see Figure 2.28). However, the production of osteocalcin/DNA was significantly greater on 3-d HA compared to the 2-d plastic control, after 14 days in culture (see figure 2.28).

Figure 2. 28: Expression of osteocalcin/DNA by MSCs cultured on 3-d porous HA compared to 2-d HA discs and 2-d plastic control, after 14 days in culture * P<0.005.



3.4 DISCUSSION

For MSCs to be successfully used to tissue-engineer bone, in addition to differentiating into osteoblasts it is necessary to grow the cells on a suitable carrier, thus as discussed in this chapter's Introduction, a porous HA scaffold was tested. The aim of Chapter 3 was to test whether MSCs isolated from human bone marrow would grow on an HA surface and whether this surface would stimulate differentiation into osteoblasts without the influence of OS, which was found to be needed for such differentiation in standard culture conditions in Chapter 2.

3.4.1 Cellular proliferation rate conclusions

3.4.1.1 2-Dimensional HA discs

3.4.1.1.1 DNA as a measure of proliferation

To test the first hypothesis in this chapter, that MSCs isolated from bone marrow will grow on a HA surface *in vitro*, the total DNA content of cells cultured on HA discs was measured over 15 days and compared with the plastic control. As discussed in Chapter 2 Discussion, although the assay does not distinguish between alive and dead DNA, as dead cells would be removed, DNA levels suggest cell number and DNA levels over time can be used to assess cell growth.

In summary, the results show that when bone marrow isolated MSCs were cultured on the 2-d plastic surface, the DNA content increased significantly over the 15-day period ($P<0.005$). Although this indicates that the cells were proliferating in this environment, as only three-time points were measured it is difficult to comment further on the grown pattern. The growth of a cell population would be expected to follow a sigmoid curve, with the proliferation rate reaching a plateau as growth becomes limited by environmental factors. Hence, these results may indicate that this point was not reached.

In contrast, there was not found to be a significant increase in DNA content of MSCs on the HA discs between days 5 and 15 in culture. However, the DNA content of MSCs cultured on HA was significantly greater than on plastic after 5 days ($P<0.05$). This suggests that over the first 5 days in culture MSCs grow more rapidly on HA, but

plateau between days 5 and 15, with the DNA content of MSCs on plastic being significantly greater by day 15 ($P<0.05$).

The study of growth of osteoblasts *in vitro* has shown that there is an inverse relationship between proliferation and differentiation (Aronow et al. 1990; Malaval et al. 1999; Oreffo et al. 1998). The maturation of osteoblasts has been defined as occurring over three phases of growth: proliferation, matrix maturation and mineralisation (Stein et al. 1990). Proliferation genes (c-fos and c-myc) have been shown to be expressed over the first 5 days of osteoblastic culture (Lian & Stein 1992) and osteoblastic proteins start to be produced after 8-10 population doublings when osteoblast progenitor cells are cultured *in vitro* (Malaval et al. 1999). Thus, during osteoblastic growth, proliferation occurs first and this reduces as the cells differentiate and produce matrix that is mineralised.

Therefore, an explanation for the observed increase in DNA (cell number) relative to the control, over the first five days, when MSCs were cultured on HA, is that this represents the initial proliferative phase. The lack of further increase in DNA over the remaining period, in contrast to the control, is consistent with the later differentiation phase of osteoblasts, at which point proliferation is reduced.

3.4.1.1.2 Alamar blue as a measure of proliferation

Cell growth was further assessed by the use of Alamar blue assay, which is an indictor of cellular redox state. As culture medium is reduced as a result of cellular metabolism, division and growth, the blue to red colour change of Alamar blue is proportional to the degree of reduction, thus indicating cellular activity. This assay has been shown to correlate well with other methods of measuring proliferation, MTT assay (Back et al. 1999) and trypan blue exclusion (Gazzano-Santoro et al. 1997). Thus it has been widely used as a quantitative measure of the proliferative activity of cells in culture (Ahmed et al. 1994; Nakayama et al. 1997). Alamar blue assay also has the advantage that it is non-toxic to cells and can be applied without cell death to cells in culture, unlike the MTT assay, with the result that it can be used serially on the same sample of growing cells over time. Furthermore, it allows measurement of cell viability, unlike cell counts or DNA assays that also include dead cells and was therefore used in my study as a further measure of proliferation.

In my study it was observed that Alamar blue absorbance was significantly greater when MSCs had been cultured for 5 days on HA discs compared with 10 days ($P<0.005$), following which point there was no significant change over the remaining culture period. Thus, cell proliferation on the HA surface peaked at 5 days *in vitro*, following which it reached a plateau, reflecting the DNA levels (see above). The plateauing is consistent with the study of osteoblastic cell growth in culture, which has been found to go through an initial proliferation phase followed by cell differentiation (Lian & Stein 1992; Malaval et al. 1999; Stein et al. 1990). Therefore my results of the growth of MSCs suggest that after 5 days on HA the proliferation rate decreased, which is consistent with osteoblastic differentiation of cells.

3.4.1.2 3-Dimensional porous HA scaffold

3.4.1.2.1 DNA as a measure of proliferation

Following seeding of MSCs on HA scaffolds, the levels of DNA were measured over 15 days *in vitro* and were found to remain constant, similarly to the results for MSCs grown on HA discs (see above). Both these results are in contrast to those for MSCs cultured on plastic and suggest that the MSCs only proliferated during the first 5 days in culture on HA. The lack of a further increase in cell number over the following 10 days in culture correlates with cell differentiation on HA, as discussed above.

DNA measurements per number of cells seeded were significantly greater for MSCs cultured on the 3-d HA scaffold as compared with the 2-d HA disc at each time point measured ($P<0.005$). This suggests that the porous nature of the scaffold, which increases its surface area, allows more rapid cell division.

3.4.1.2.2 Alamar blue as a measure of proliferation

Alamar blue absorbance measured on MSCs cultured on HA scaffolds were found to be significantly greater than on the plastic scaffold control ($P<0.005$). However, the absorbance levels from cells on the plastic control were very low and immeasurable at 15 days. This impliesthat MSCs were unable to grow on this substrate and, as a result of this finding, plastic scaffolds were not used for the remaining experiments in this chapter.

The proliferation rate indicated by the level of Alamar blue absorbance by MSCs did not change significantly over 15 days in culture on the HA scaffold. An explanation of this is that, as the DNA levels were constant on the scaffolds and greater than on the corresponding discs over the culture period and as the Alamar blue results for MSCs grown on HA discs was maximised after 5 days in culture, the Alamar blue results on the HA scaffold may have been greatest during the first 5 days in culture in order to achieve the increased levels of DNA by day 5. Further experiments would be needed to measure Alamar blue levels over the first 5 days in culture on HA scaffolds to test this theory.

In response to the hypothesis stated at the beginning of this section, MSCs were able to grow and proliferate on an HA surface and HA scaffold *in vitro*.

3.4.2 Osteoblastic differentiation on 2-Dimensional HA discs

To test the second hypothesis of this chapter, that MSCs differentiate into osteoblasts without the influence of OS when cultured on HA *in vitro*, MSCs were cultured on HA discs and osteoblastic differentiation was assessed by comparing the cell morphology and osteoblastic protein production with MSCs cultured on the control surface.

3.4.2.1 Conclusions from SEM results

SEM was used to assess the change in cellular morphology of the MSCs cultured on the HA discs over the 14 day period compared with the controls. It was observed that MSCs attached to the HA via cellular processes and that the number of cells increased over the culture period until, by day 14, the HA was covered in cells. This confirms that HA is a viable surface for the growth of MSCs, reflecting the proliferation results.

After one day in culture on HA, the cells were long spindle-shaped, similar to MSCs cultured in standard medium on therminox and resembling fibroblasts. However, after 7 days in culture on HA, the cell shape changed with cells becoming brick-shaped, which is characteristic of osteoblasts (Aubin & Liu 1996; Liu et al. 1997; Lomri et al. 1988; Malaval et al. 1999). In contrast, although grown under the same culture conditions, MSCs on the control surface remained fibroblastic in shape (see Results).

Similarly to the observed cellular morphology on HA, MSCs cultured with OS on therminox became cuboidal shape, as suggested in Chapter 2 to reflect osteoblastic differentiation. However, cells cultured on HA possessed multiple cell processes, attaching them to the surface and neighbouring cells. The observed increased density of cell processes on HA suggested that MSCs preferentially adhere to HA. Furthermore, when MSCs were cultured on the HA surface they seemed to embed the edges of their cell membrane into the HA. As there is embryological evidence that cell attachment is necessary for differentiation (Pechak et al. 1986), this interaction between MSCs and HA surface may be an initiating factor for osteoblastic differentiation.

3.4.2.2 Use of proteins to assess osteoblastic activity

As ALP, osteopontin and osteocalcin proteins are produced by osteoblasts, as a measure of osteoblastic differentiation the levels of these proteins produced by the cells cultured on HA discs was compared with on the control surface. In order to test the hypothesis that MSCs differentiated into osteoblasts without the influence of OS when cultured on HA, OS was not used in any of the protein assay experiments.

Protein levels measured in the experiments of this chapter were divided by the total DNA content of the cells to standardise the readings and allow comparison, as discussed in Chapter 2.

3.4.2.2.1 Measurement of ALP protein production

ALP is a marker of osteoblast progenitor cells and osteoblasts, thus production of this protein was used as an indicator of osteoblastic differentiation (see Chapters 1 and 2). Initially, when HA discs and controls were seeded with 25,000 cells measurement of ALP levels after 5, 10, 15 and 30 days in culture did not indicate a significant difference between the two groups.

As a result of this, in order to expand any difference between the two groups, the experiment was repeated using a larger cell density (250,000 cells) over a longer culture period (28 days). This resulted in the measurement of a significantly greater production of ALP by the MSCs cultured on HA compared with the control, after both

14 ($P<0.05$) and 28 days ($P<0.005$). The production of ALP by MSCs on HA increased over the 28 days, suggesting that either more cells were stimulated to differentiate or that individual cells increased their production of ALP. However, MSCs seeded on the control continued to produce low levels of ALP over the same time. As MSCs seeded on HA produced significantly larger amounts of ALP, suggests that the cells were continuing to differentiate into osteoblasts.

3.4.2.2.2 Osteopontin protein production

Production of osteopontin extracellular protein was measured as a second marker of osteoblasts (Nakase et al. 1994), (see Chapters 1 and 2). A higher initial density of cells was used for this assay, as osteoblasts produce extracellular proteins once the cells have reached confluence.

Osteopontin production was significantly greater by the samples of 250,000 cells cultured on HA discs than by cells on the control surface, after 14 days ($P<0.005$). Furthermore, the production of osteopontin on the HA increased significantly over 28 days. Both the higher initial level of osteopontin, as compared to the control, and the continued production on HA are indicators of expression of osteoblastic phenotype by the MSCs. As osteopontin is an osteoblastic extracellular matrix protein, this is may reflect growth of the osteoblasts into matrix maturation phase.

3.4.2.2.3 Osteocalcin protein production

Osteopontin is not totally specific to osteoblasts, as noted in Chapter 2, thus osteocalcin levels were also measured. As osteocalcin is an extracellular protein that binds calcium to the matrix thereby facilitating mineralisation, it is a more precise marker for osteoblasts. When osteocalcin levels were compared between the MSCs cultured for 14 days on HA discs and the control, those on HA produced osteocalcin, which was not detected on the control. This confirms the presence of active osteoblasts entering the mineralisation phase, resulting from MSCs culture on the HA and lack of such production on the control is consistent with the maintenance of undifferentiated MSCs in this culture.

The notable change in cellular morphology and the production of proteins by cells is consistent with that of the osteoblastic phenotype, in comparison to the control. This

therefore supports the first part of the second hypothesis of this chapter that MSCs differentiate into osteoblastic cells without the influence of OS when cultured on HA discs *in vitro*.

3.4.3 Osteoblastic differentiation on 3-d porous HA scaffolds

In order to test whether MSCs would also differentiate into osteoblasts on porous HA scaffolds, throughout the following experiments cells were cultured in standard culture medium without additional OS.

3.4.3.1 Light microscopy as a assessment of morphology

On examination under light microscopy, the MSCs were observed to attach themselves and grow on all surfaces of the porous HA, confirming it as a useful substrate for MSC culture. The larger surface area allowed a greater number of cells to attach and grow in a smaller volume. At higher magnification, after 5 days in culture it was observed that the shape of individual cells was fibroblastic and remained similar in shape to the original MSCs.

A control scaffold of polyethylene plastic foam, with the same macro-porous structure as the porous HA was chosen, on which it was intended to culture MSCs and against which the results from HA were to be compared. However, few cells could be seen on the scaffold under light microscopy staining and, as stated above, the proliferation rates of MSCs on this scaffold were very low. It was therefore concluded that the cells would not grow on this scaffold and the results could not be used, as intended, as a control for the porous HA. Hence, instead, the levels of osteoblastic markers produced by MSCs cultured on 3-d HA scaffolds were compared with those of 2-d HA, which acted as a control for the 3-d structure.

3.4.3.2 Conclusions from SEM

As was observed on HA discs under SEM, MSCs attach to the surface of the HA scaffold by multiple processes. It was observed that the cells cultured on the scaffolds changed shape over the 14-day culture period became cuboidal and resembled osteoblasts (Aubin & Liu 1996) (Benayahu et al. 1994). Round cells, suggestive of active osteoblasts were also observed (Yoshikawa et al. 1996). The cell density was

observed to increase greatly over time as scattered cells proliferated to cover completely the surface with osteoblastic cells by day 14.

3.4.3.3 Conclusions from TEM

Under TEM, MSCs cultured on HA scaffolds were observed to contain intracellular rough endoplasmic reticulum and mitochondria after 28 days, evidence of metabolically active cells (Bab et al. 1988b). Early organised collagen fibrils were observed adjacent to cells, demonstrating that the cells produced collagen and this was arranged into a matrix. Both observations suggest the presence of functioning osteoblasts.

3.4.3.4 ALP protein production

ALP production increased significantly over the 15-day culture period. As ALP is a marker of the osteoblast phenotype, this suggests that MSCs differentiated into osteoblasts over 15 days on HA scaffolds.

However, the levels of ALP were significantly lower on porous HA compared to HA discs. As discussed in the Introduction, materials on which cells are grown alter the cell physiology, thus, although both HA substrates should have the same composition, surface roughness and energy, the variation in topography between disc and porous scaffold may influence the production of ALP.

Using these ALP results alone as an indication of osteoblastic differentiation, suggests that MSCs differentiated to a greater extent on HA discs compared to HA scaffolds. However, for a bone graft substitute to be useful for implantation to heal a bone defect, it needs to enable nutrients to diffuse through it to maintain living cells, which can form bone. In this experiment the HA scaffold would allow this and the protein results indicate that MSCs on this HA scaffold produced increasing levels of ALP indicative of osteoblastic differentiation.

3.4.3.5 Osteocalcin protein production

Osteocalcin was not found to be produced to any greater extent by MSCs cultured on porous HA as compared to those cultured on HA discs, although production on both

forms of HA was significantly greater than on the control ($P<0.005$). MSCs grown on an HA scaffold produce osteocalcin, confirming the results observed under TEM, that the cells are differentiating into osteoblasts and producing extracellular matrix proteins that have the potential for mineralisation.

In conclusion, MSCs cultured on both HA discs and the HA scaffold produced significantly greater amount of osteoblastic proteins compared to the control. This suggests that it is the HA rather than the 3-d environment that is stimulating osteoblastic differentiation. Although the production of ALP was less on the scaffold compared to the disc, MSCs cultured on both HA substrates were shown to produce osteocalcin, indicating differentiation into active osteoblasts *in vitro*. These results confirm the second hypothesis and suggest that the porous HA scaffold would be favourable for use in tissue engineering of bone.

CHAPTER 4

A Novel Bioreactor for the Tissue Engineering of Bone

4.1 INTRODUCTION

4.1.1 Background to this chapter

Although bone has a good capacity for healing, critical sized bone defects will not heal without intervention, as discussed in Chapter 1. Bone tissue is mechanical supportive thus a 3-d structure is needed to repair such defects. The advantage of tissue engineering, as discussed in Chapter 1, is that biological material is used to repair these defects. These tissue-engineering principles could be applied to a porous scaffold that should allow cell growth through it and result in a suitable 3-d cellular structure. The capacity for healing would be increased by the presence of osteoinductive cells within the tissue grafts. These constructs could then be packed into a bone defect *in vivo*.

In the experiments in Chapter 2, it was shown that MSCs could be isolated from human bone marrow aspirates and had the potential to differentiate into osteoblasts when cultured *in vitro* with OS. In Chapter 3 it was shown that MSCs could be successfully cultured on porous HA in stationary culture and that the cells differentiated into osteoblasts without the addition of OS, confirming that HA scaffolds could be used as a substrate for tissue-engineered bone. Further to this, this chapter investigates whether the use of a novel bioreactor increases the potential of MSCs on a HA scaffold to be used for the tissue engineering of bone.

4.1.2 The use of bioreactors

A bioreactor is a tissue culture vessel in which culture conditions can be controlled over the entire culture period. Bioreactors have been used by the pharmaceutical and biotechnology industries to enable the production of large quantities of cells, bacteria, and protein products such as factor VIII, erythropoietin, antibodies and vaccines. Research has increased considerably in this area over the last decade, however the applications for bioreactor use in the field of tissue engineering are just in their infancy.

4.1.3 Advantages of bioreactor culture

Cells grown in stationary tissue culture require serial passaging to produce large numbers, which is a labour intensive process. In contrast, bioreactors allow continuous culture, which produces a larger number of cells than in static culture. Furthermore, by creating a more efficient environment for the transfer of nutrients and waste, bioreactors can support higher growth rates than can be maintained in static culture conditions.

4.1.4 Types of bioreactor

Bioreactors can be classified into three groups: Homogenously agitated bioreactors, Agitated systems and Heterogeneous bioreactors.

Homogenously agitated bioreactors aim to maintain a constant uniform physiochemical environment, for example roller bottles, which are used to produce vaccines and cell suspensions.

Agitated bioreactors allow a uniform environment within the bulk liquid, although gradients occur across the constructs. These systems include Rotating Wall Vessels (RWV) as first designed by NASA to simulate the effects of microgravity on the muscular skeletal system, as experienced in space (Klement & Spooner 1993). These bioreactors suspend cells by rotating the inner of two chambers and inducing secondary airflow in the outer chamber, keeping cells in constant freefall of microgravity (Schwarz et al. 1992). This reduces mechanical stresses allowing cells to grow unrestricted by gravity.

As a result, agitated bioreactors have been used by NASA to protect cells from shear forces of launch and landing, allowing cell culture to be studied in space. Furthermore, when these systems were tested on Earth, it was shown that cells formed aggregations and structures resembling tissue and consequently, RWV have been studied using 3-d cell models for tissue engineering cartilage (Freed et al. 1993) and bone (Granet et al. 1998). Under these conditions it was found that the proliferation rate of cells increased, as did protein production specific to the cell types.

RWV have also been shown to simulate the *in vivo* cellular environment as cells produce proteins otherwise not produced *in vitro*. For example, human colon carcinoma cells produce carcinoembryonic antigen when cultured in this environment (Jessup et al. 1997). Therefore, this culture method supported the tumour cell function that normally only occurs *in vivo*, allowing possible treatments for certain cancers to be studied *in vitro*.

Heterogeneous bioreactors comprise the remaining systems that have been designed for the culture of specific cell types. These use scaffolds, membranes and controlled flow rates to produce gradients with the aim of generating tissue, such as dermal skin and vascular grafts. It is within this group that the bioreactor used in my study can be classified.

4.1.5 Response of bone to environmental factors

The relationship between the structure and function of bone was first suggested by Julius Wolff "Every change in the function of a bone is followed by certain definite changes in the internal structure architecture and external conformation in accordance with mathematical principles" (Wolff 1892).

Since then, it has been observed that bone atrophy occurs after prolonged periods of bed rest. Hence, mechanical loading of the skeleton is crucial for bone development and metabolism, with increased mechanical loading increasing bone formation and decreasing bone resorption (Morey & Baylink 1978). Conversely, the absence of mechanical stimulation causes a reduction in bone matrix protein production, mineralisation and bone formation (Sessions et al. 1989). At a cellular level, load-induced bone modelling has been shown to be mediated by prostaglandin production, which recruits progenitor cells and osteoblasts (Pead & Lanyon 1989). Therefore, mechanical stimulation affects bone remodelling.

More recently, the importance of fluid flow on bone metabolism has been investigated. The effect of altering the fluid flow through a canine tibia was studied using a venous tourniquet model. Increasing the venous pressure in the tibia increased the extracellular

fluid flow resulting in increased new bone formation, indicating that extravascular perfusion enhances bone formation (Kelly & Bronk 1990).

In cortical bone, interstitial fluid flows from capillaries in Haversian canals through the canuliculi-lacunar complex to supply osteoblasts and osteocytes, driven by hydrostatic pressure as well as mechanical loading. The Haversian canals are 20-30μm in diameter and extend along the longitudinal length of cortical bone and lacunae continue from Haversian lamellae, measuring 11 - 14μm in transverse diameter and extending in the long axis of the diaphysis for an average length of 24μm (Qin et al. 1999). The size of the diameter of the micro-pores within the HA scaffold used in my thesis, reflected the structure of cortical bone (see Chapter 3, Introduction).

Variation in interstitial fluid flow through bone effects mechanical loading signals altering cellular activity. The effect of fluid flow on osteoblasts has been investigated *in vitro* and increased flow results in the release of autocrine and paracrine factors that stimulate osteoblast activity. Prostaglandin E_2 (PGE_2) production by osteoblasts *in vitro* has been shown to increase when cells are exposed to shear fluid flow (Reich & Frangos 1991). The increase in PGE_2 also mediates increases in secondary messengers cyclic adenosine monophosphate (cAMP) and inositol trisphosphate (IP_3), which have also been shown to be up-regulated by fluid flow specifically in osteoblasts (Reich et al. 1990; Reich & Frangos 1991).

Increases in these secondary messengers cause the release of intracellular calcium from endoplasmic reticulum (Hung et al. 1995). Intracellular calcium modulates actin cytoskeleton, which stimulates gene expression of c-fos, produced by proliferating osteoblasts and COX-2, a key enzyme in the production of prostaglandins. Intracellular calcium is also important in the formation of connections between the cytoskeleton and integrins, which aid osteoblastic interaction with the extracellular matrix and in turn regulate cell function and differentiation (Chen et al. 2000). Intracellular calcium is therefore vital in mediating the response of osteoblasts to fluid flow.

Nitric oxide (NO) stimulates osteoblast activity and inhibits osteoclast resorption, as levels of NO are undetectable in stationary rat calvarial cell culture, but production of

NO rapidly increases in response to fluid shear stress (Johnson et al. 1996). Hence, this is a further mechanism by which osteoblast activity is affected by fluid flow. It has also been shown that osteopontin gene expression increases in response to fluid forces (Owan et al. 1997).

In summary, a culture environment where fluid flow is used to reproduce the physiological environment may increase osteoblast activity and the generation of bone extracellular matrix via the pathways outlined.

4.1.6 Design of the bioreactor

In short, the aim of my bioreactor was to allow MSCs to grow through an HA scaffold, encouraging MSCs to differentiate into osteoblasts and produce an extracellular matrix by perfusing the scaffolds with a pulsatile flow of medium. Each of the criteria discussed below were considered in the design of my bioreactor:

- Grow viable cells
- Encourage cell proliferation
- Stimulate MSCs to differentiate into osteoblasts
- Allow extracellular matrix production
- Encourage cellular penetration of scaffold

4.1.6.1 Grow viable cells

Various physical and environmental criteria need to be met for the successful growth of cells *in vitro*. At an elementary level, these criteria include a supply of glucose, amino acids, electrolytes, water, oxygen and carbon dioxide (CO_2). Insufficient supply of oxygen can become the limiting factor for cell growth within a scaffold, as oxygen has to diffuse through the scaffold in static culture. As a result, cells at the centre of the construct are susceptible to low oxygen levels, which leads to cell apoptosis. This was avoided in my bioreactor, as the porous scaffolds were perfused with oxygenated medium.

In static cell culture the medium was changed twice a week, with the aim of maintaining nutrient levels. However, in this bioreactor design, medium flowed through the scaffold culture, providing a constant supply of sterile nutrients. As medium flowed through the system once, the reservoir was refilled every 2 – 3 days

and the outflow container emptied, also preventing contamination or denaturing of constituents within it. It is also necessary for any feeding or sampling from the bioreactor to be aseptic to prevent contamination and infection.

The appropriate environment to stimulate cell growth was achieved by placing the bioreactor system within an incubator at 37°C and 5% CO_2 under sterile conditions. An open system was used, allowing air into the feeding reservoir to displace the used fluid and ensure that the medium remained oxygenated. In addition to this, silicone tubing used would allow diffusion of gas into it and, to facilitate this, the tubing was placed under the fan within the incubator.

4.1.6.2 Encourage cell proliferation

Ideally tissue-engineered bone would only require a small sample of autologous cells, thus avoiding the need for large quantities of donor tissue to be harvested. Although MSCs are infrequent in bone marrow they have a large capacity for proliferation and this capacity needs to be utilised to expand the cell numbers, for MSCs to be used in tissue engineering. This can be achieved in monolayer culture and human MSCs have been shown to maintain their phenotype for more than 20 passages (Jaiswal et al. 1997), but this process is both time consuming and labour intensive. As noted, the dynamic conditions of a bioreactor increase the efficiency of mass transfer of nutrients, so allowing greater continuity of cell growth. Thus, as MSCs can potentially be expanded in my bioreactor, these cells were studied rather than differentiated osteoblasts.

In static culture conditions, the cell growth rate is dependant on diffusion for the supply of oxygen and nutrients. As the cells proliferate, their mass increases and the corresponding scaffold porosity decreases, resulting in a limited supply of oxygen and nutrients reducing cell growth. Therefore, if the supply of nutrients and removal of waste can be maintained over time by increased mass transfer to all parts of the scaffold, which it is suggested would be achieved by culturing in my bioreactor system, cell growth can continue unabated resulting in the formation of tissue.

The aim of my bioreactor design was to increase cell proliferation by addressing these limitations of static culture. RWV systems allow cell proliferation to continue for

longer periods of time than cells in static culture (Granet et al. 1998) and a perfusion system has been shown to increase osteosarcoma cell proliferation in culture (Mueller et al. 1999). My bioreactor design maintains a constant supply of nutrients to the cells through the scaffold, by perfusing with fresh oxygenated medium with the aim of maintaining cell proliferation.

4.1.6.3 Stimulate MSCs to differentiate into osteoblasts

In Chapter 3 it was shown that MSCs could be cultured in static conditions on HA scaffolds, with a similar structure to trabecular bone and micro-porous size similar to the cortical bone system, and that MSCs differentiated into osteoblasts. Although there was osteoblastic differentiation using this culture method, greater osteoblastic activity producing extracellular matrix, is required in order to generate bone tissue. In this study osteoblastic differentiation and function of MSCs on HA in the bioreactor will be compared with MSCs seeded on the same HA scaffolds and cultured in stationary tissue culture conditions, referred to as static conditions in this chapter.

Trabecular bone is well perfused by blood and within cortical bone osteoblasts line Haversian canals, which contain capillaries. Thus, osteoblasts and osteocytes are supplied with oxygenated blood *in vivo*. Additionally it has been shown that, preferentially MSCs differentiate into osteoblasts in well-oxygenated tissue (Bab et al. 1988b). The perfusion of well-oxygenated medium was therefore passed through the HA-MSC culture in this bioreactor to encourage osteoblastic differentiation.

It has been shown that fluid flow stimulates osteoblastic function *in vivo*, as described above. Furthermore, the perfusion of medium increased osteosarcoma cell viability and protein production on 3-d collagen after 21 days *in vitro* (Mueller et al. 1999). Therefore, the flow of medium through the HA scaffold recreates the physiological environment which is hypothesised in this chapter, to stimulate cell signalling resulting in an increase in osteoblast differentiation and activity.

It has been shown that oscillatory fluid flow increases the biological response of osteoblastic cells, measured by increased intracellular calcium and osteopontin gene expression (You et al. 2001). In living bone, the flow of fluid results from vascular pressure, but also varies with mechanical loading. Thereby, with walking, the

physiological flow produced is pulsatile and this was simulated in the bioreactor by the use of a peristaltic pump.

In a study of osteoblasts on a scaffold it was observed that, although initially cell numbers increased with flow rate, a flow greater than 1ml/min was detrimental to cell viability (Porter et al. 2001). This pattern is explained by the initial increase in flow increasing the supply of nutrients and removal of waste from the cells, but as flow rate increases further the shear forces on the cells increase correspondingly preventing further cell proliferation. Therefore, the flow rate of 0.1ml/min was initially used in my study to enable cell proliferation.

4.1.6.4 Allow extracellular matrix production

It is proposed that in my perfusion bioreactor, the flow of fluid will stimulate differentiation and activity of osteoblasts, thus increasing the production of extracellular matrix. Furthermore, the slow perfusion rate will allow the organised deposition of extracellular matrix on the HA scaffold. Since HA is the major inorganic component of bone, it would provide a suitable substrate for the facilitation of osteoblastic extracellular matrix. As a result of the porous nature of the scaffold, the extracellular matrix should form within the pores, producing a tissue-like structure.

4.1.6.5 Encourage cellular penetration of scaffold

The porous structure of the HA allows MSCs to grow on surfaces through the scaffold. However, penetration of the scaffold by cells needs to be stimulated by the culture method or cells are likely to remain on the top of the scaffold. The flow of medium through the porous scaffold may stimulate cell migration, increasing cellular penetration of the scaffold. Furthermore, culture in this bioreactor system should ensure a supply of nutrients to the centre of the scaffold, thereby allowing cells to remain viable throughout the depth of the scaffold.

4.1.7 In this chapter

MSCs have been shown to be precursors of osteoblasts and are present in bone marrow (see Chapter 1). As a result they can differentiate down the osteogenic lineage and potentially produce bone. As MSCs also have a great propensity to replicate and proliferate, these cells are important in fracture healing.

The aim of this chapter was to design a novel bioreactor for the culture of MSCs on HA scaffolds, which would encourage the differentiation of MSCs and production of bone extracellular matrix. The design of the bioreactor utilised the theory that if nutrients flowed over cells through a porous scaffold, it may increase the differentiation of MSCs into osteoblasts at a greater rate than in static culture conditions. This was studied by observing the cell morphology under SEM (as in Chapter 3, measuring osteoblastic mRNA and protein production and assessing the presence of an extracellular matrix under TEM.

In addition to providing efficient mass transfer of nutrients, the design of my bioreactor should increase the penetration of MSCs through the porous scaffold and allow a uniform distribution of cells on a 3-d scaffold. Thus improving the constructs' 3-d cellular structure and making it ideal for tissue engineering applications. Evidence of cell penetration through the HA scaffolds was assessed by cross-sectioning the scaffolds and counting cell numbers under SEM.

4.1.8 Hypothesis

This chapter investigates the use of a novel bioreactor system for the culture of MSCs on HA scaffold in 3-d environment. Therefore the hypotheses tested in this chapter were:

1. MSCs, cultured on porous HA in the bioreactor, penetrate through the scaffold to a greater extent, than when cultured in a static environment.
2. MSCs, cultured on porous HA in a perfusion system of the bioreactor, differentiate into osteoblasts and produce extracellular matrix.

4.2 MATERIALS and METHODS

4.2.1 Materials

Cell culture materials as Chapter 2

Porous HA block as Chapter 3

Standard culture medium used in the bioreactor, as detailed in Chapter 2, containing:

Dubeccos Modified medium-low glucose (Sigma D6429)

10% foetal calf serum (Sigma F7524)

Penicillin 50U/ml (Sigma (Sigma P0906)

Streptomycin 50µl/ml (Sigma (Sigma P0906)

Bioreactor:

Pump (Watson Marlow 102R)

Silicone tubing, 3.2mm wall thickness (Merck 910 0016 016)

RNA materials as Chapter 2

ALP & DNA assays as Chapter 2

Type I procollagen radio-immunoassay (Orion Diagnostica 68569)

4.2.2 Cell culture

The MSCs were isolated from ten human bone marrow aspirates and cultured in standard monolayer conditions up to passage 3, as described in Chapter 2. No osteogenic supplements were used during any part of this chapter's experiments.

4.2.3 Seeding cells on scaffold

The MSCs were trypsinised, counted using a haematocytometer and $1x10^6$ cells were seeded in 0.25ml of medium onto each porous HA scaffold, as described in Chapter 3. The seeded scaffolds were incubated at 37°C, 5% CO_2 for 2 hours to allow the MSCs to adhere to the HA, but without the medium evaporating as this would cause the cells to die. This was to allow the cells to adhere to the surface and prevent them from being washed off the scaffold during transfer. After this half the scaffolds were transferred to the bioreactor chamber and submerged in medium. While the remaining HA scaffolds

were submerged in medium in well-plates and cultured in the same incubator. This method was used to set up each experiment in this chapter, with the same number of scaffolds being cultured in the bioreactor or static culture.

4.2.4 Bioreactor culture

The HA scaffold's porous structure provided a large surface area for the cells to cover and in my bioreactor all the cells on the scaffold were in contact with medium, as medium flows through the scaffold. The flow rate was controlled by a peristaltic pump and could be varied, but a rate of 0.1ml/min (144ml/24hr or 6ml/hr) was tested, as discussed in the Introduction to this chapter. The medium was pumped through silicone tubing from a reservoir, through the bioreactor chamber and out into an outflow container. The flow of medium maintained a fresh supply of nutrients and the removal of waste.

The whole system was cultured in aseptic conditions within an incubator at 37°C and 5% CO_2. The reservoir of medium within the incubator was warmed to 37°C and refilled every two days. This maintained a constant flow of medium, but preventing large volumes of stagnant medium being held in the incubator, which would be susceptible to the risk to contamination (see figure 3.1).

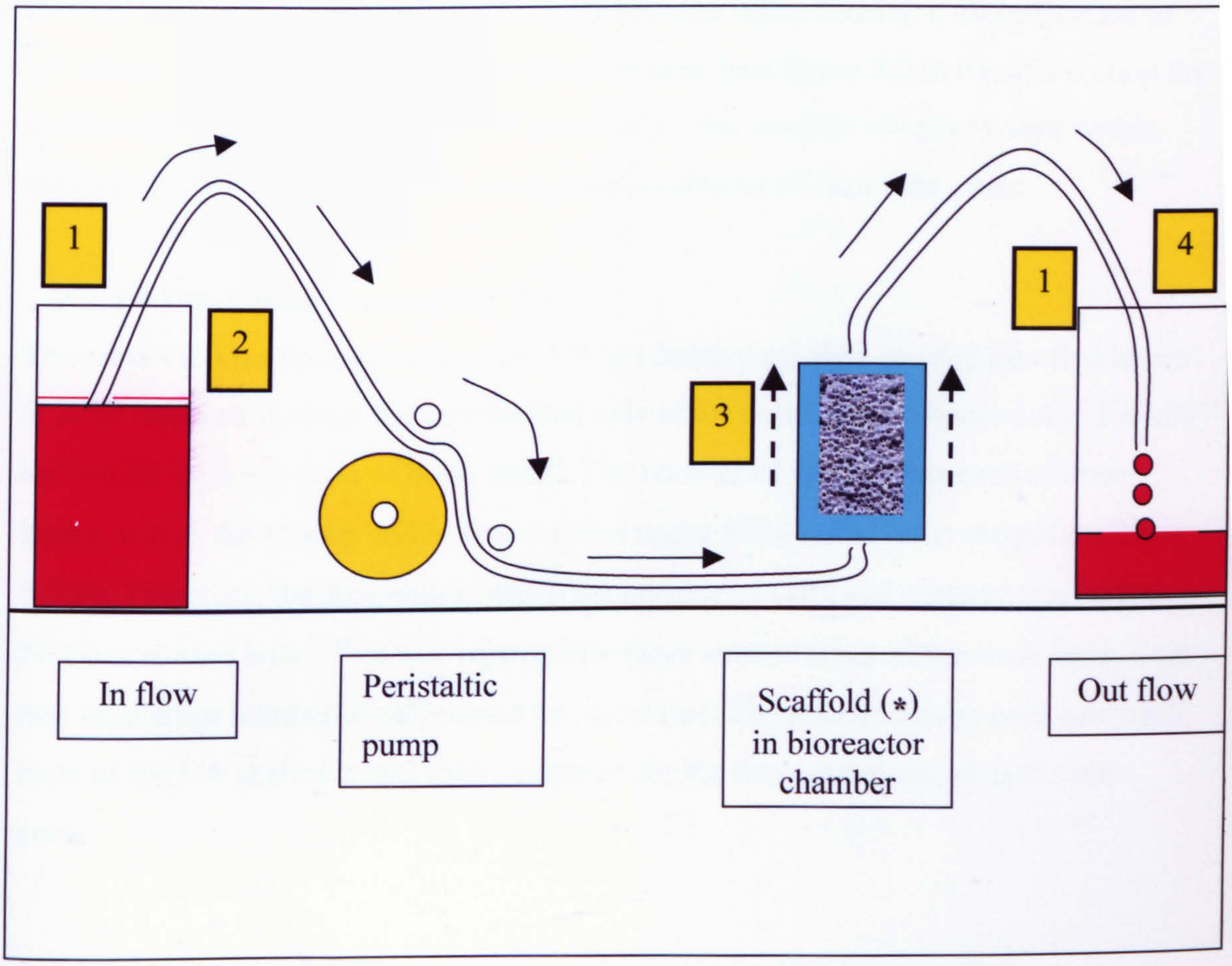
4.2.5 Assessment of penetration of cells through scaffold

4.2.5.1 SEM processing and analysis

To assess the penetration of cells through the HA scaffolds in the bioreactor as compared with static culture, cells from 6 patients' aspirates were each seeded on 4 HA scaffolds, as described above. Each of these scaffolds was then cultured for 7 or 14 days, in either the bioreactor or in static tissue culture. Thus for the SEM experiments to assess cell penetration, there were a total of 6 scaffolds cultured in each condition, at each time-point, resulting in a total of 24 scaffolds. Following each culture period the scaffolds were processed for SEM (see Chapters 2 and 3 Methods). The tops of the scaffolds were initially observed under SEM.

Figure 3. 1: Diagram of Bioreactor System

1. Air inlets into both in-flow and out-flow flasks
2. Feeding flask containing sterile medium
3. Chamber barrel containing material with cells on scaffolds
4. Outflow flask to collect used medium.



* *HA cell scaffold used in chamber*

4.2.5.2 HA in cross section

The processed HA scaffolds were then cut centrally, through the longitudinal axis, from top to bottom using a diamond circular saw. This enabled the penetration of the cells through the cross section of the HA to be determined. The cut sections were remounted uppermost on metal stubs, recoated with gold/palladium for 2 minutes and observed under SEM. The total number of cells present within an 855μm width, through the depth of the cross section were counted under SEM at a magnification of 150 times, at which individual cells would be seen (see figure 3.2 in Results section for an illustration of this). To assess cell penetration, the number of cells present within the cross sections was compared for the two conditions at each time-point.

4.2.5.3 Penetration through the HA

The cross sections through each of the HA scaffolds were then divided into five layers at 2mm intervals through the longitudinal axis of the sections (see figure 3.4 in Results section for an illustration of these areas). The number of cells within each of these layers, within the 855μm width, was counted under SEM at the same magnification as before. Therefore, the area within which the number of cells was counted was 855 x 2000μm at each layer. This was repeated for three similar areas within each layer, such that an average number of cells could be calculated. The penetration by cells into each layer of the HA scaffolds was then compared for the two conditions, at each time-point.

4.2.6 Measures of differentiation of MSCs in the bioreactor

4.2.6.1 SEM

The MSCs cultured in the bioreactor were assessed for osteoblastic differentiation initially by observing the morphology of the cells under SEM after 7 and 14 days, as compared with control cells grown on the same scaffold in static culture. Five HA blocks were cultured, as described above, for each condition. The medium for the cells in static culture was changed twice a week. After the culture periods were completed the scaffolds were processed for SEM. Following this cells on the scaffolds were observed under SEM and recorded.

4.2.6.2 TEM

The ultrastructure of the cells and extracellular matrix production was compared for MSCs cultured in the bioreactor with the control conditions, using TEM. For this, HA scaffolds were again seeded with $1x10^6$ MSCs and cultured in either condition for both 14 and 28 days, following which the scaffolds were processed for TEM (see Chapter 3 Method). Two patient's aspirates were used for each condition at each time point. Following initial TEM processing and embedding in resin, 1mm sections through the HA scaffolds were observed under light microscopy. This allowed an overall comparison between the two conditions. Areas observed to contain cells were then re-embedded in resin and thin sections were cut using an ultra-microtome diamond knife. The thin sections were placed on copper grids. The HA samples for each day and condition were then observed under TEM and photographed.

An energy dispensive x-ray analysis (EDAX) probe was used to assess the chemical components of deposits seen between cells.

4.2.6.3 Messenger RNA expression by RT-PCR

The presence of specific mRNA sequences was examined within MSCs cultured on HA in the bioreactor for 1, 2 & 4 days, and similarly in the static control. After each culture period cells were removed from the scaffolds by incubating them in 2ml of 10% trypsin for 10 minutes at 37°C. Then the cells were washed with phosphate buffered saline and centrifuged in an additional 10ml of medium. The supernatant was removed from the cell pellet and these were stored at –70°C. Following thawing the RNA was extracted from the samples and analysed by reverse transcriptase polymerase chain reaction (RT-PCR) (see Chapter 2 Method). The RNA sequences probed for were ALP, osteopontin, Bone sialoprotein (BSP) and osteocalcin, known markers of osteoblasts, and Cbfa-1, suggested to be a marker of osteoblast precursor cells. For details of the gene sequences except BSP, refer to Chapter 2 Method.

BSP: 5' AAC GAA GAA AGC GAA GCA GAA GTG 3'

3' CTG ACC ATC ATA GCC ATC GTA GCC T 5'

(Phinney et al. 1999)

4.2.6.4 ALP protein assay

The protein readings were standardised for each sample as before, by dividing protein levels by total DNA content, assayed by Hoechst assay (as described in Chapter 2). The assay used measured the levels of ALP by the amount of p-nitrophenol that had been cleaved from p-nitrophenol phosphate (see Chapter 2 Method).

MSCs from 8 patients' were cultured on 8 porous HA in the bioreactor and in static culture for 1, 5, 10 & 15 days. The resulting ALP/DNA results were compared between the two conditions at each time point.

4.2.6.5 Type I procollagen protein assay

When collagen type I is derived from type I procollagen the carboxyterminal propeptite group (PICP) is removed. Thus, the concentration of PICP is proportional to recently synthesised type I collagen and bone activity (Nacher et al. 1999). The amount of PICP in each sample was measured using a competitive radioimmunoassay, where a known amount of I^{125} labelled PICP was added to the samples and PICP rabbit antiserum antibody was then added. The labelled and unlabelled antigens compete for binding sites on the antibodies, thus the bound labelled antigen was inversely proportional to the initial amount of unlabelled PICP in the samples. The amount of labelled bound PICP was measured using a gamma counter and the actual PICP measurements were calculated from a standard curve of known concentrations.

The amount of PICP/DNA was measured for MSCs cultured on HA in the bioreactor for 5, 10 & 15 days and compared with the results from similar scaffolds in static culture. MSCs from 6 patients' aspirates were used in each group.

4.2.7 Statistical analysis

The results from the culture in the bioreactor were compared to the control. The results for the assessments of cell penetration into the HA scaffolds and differentiation of MSCs using ALP and PICP were not found to follow a normal distribution, consequently non-parametric statistical tests, namely Mann Whitney U, were used to assess the significance between the two groups.

4.3 RESULTS

4.3.1 Penetration of cells through HA scaffold

4.3.1.1 Assessment of cell numbers in cross section

The total number of cells present on the cross-sectioned surface of the HA scaffolds was compared from those cultured in the bioreactor compared with static conditions. The number of cells within the cross sectional surface was determined by counting cells within an 855μm width (see figure 3.2). The number of cells penetrating through the HA scaffolds that had been cultured in the bioreactor was significantly greater than for static culture, after both 7 & 14 days ($P<0.05$), (see figure 3.3).

Figure 3. 2: A cross-sectioned porous HA scaffold illustrating the 855μm width within which the number of cells were counted, under SEM. The cell number within this width was compared between bioreactor and static culture conditions. Bar = 1mm

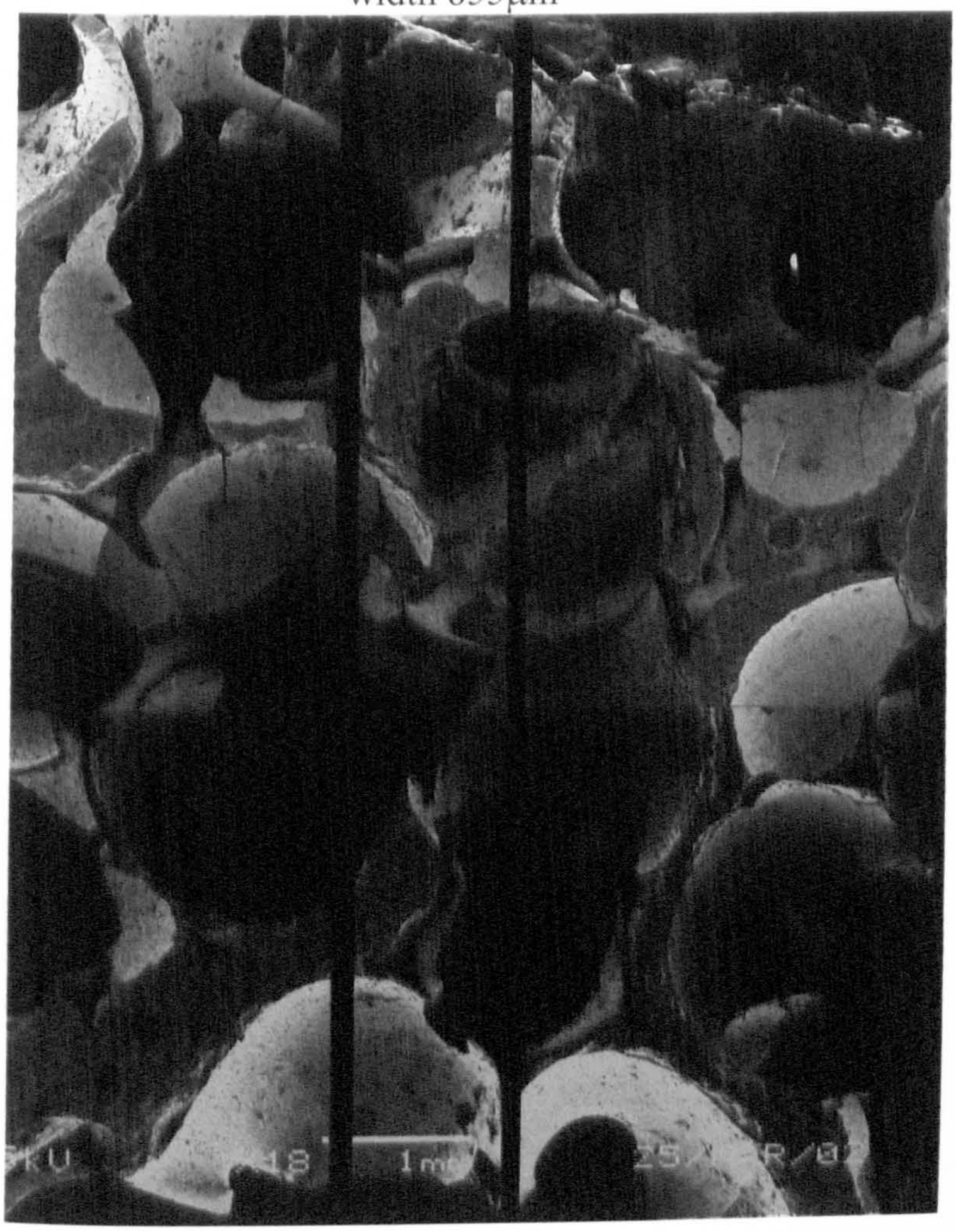
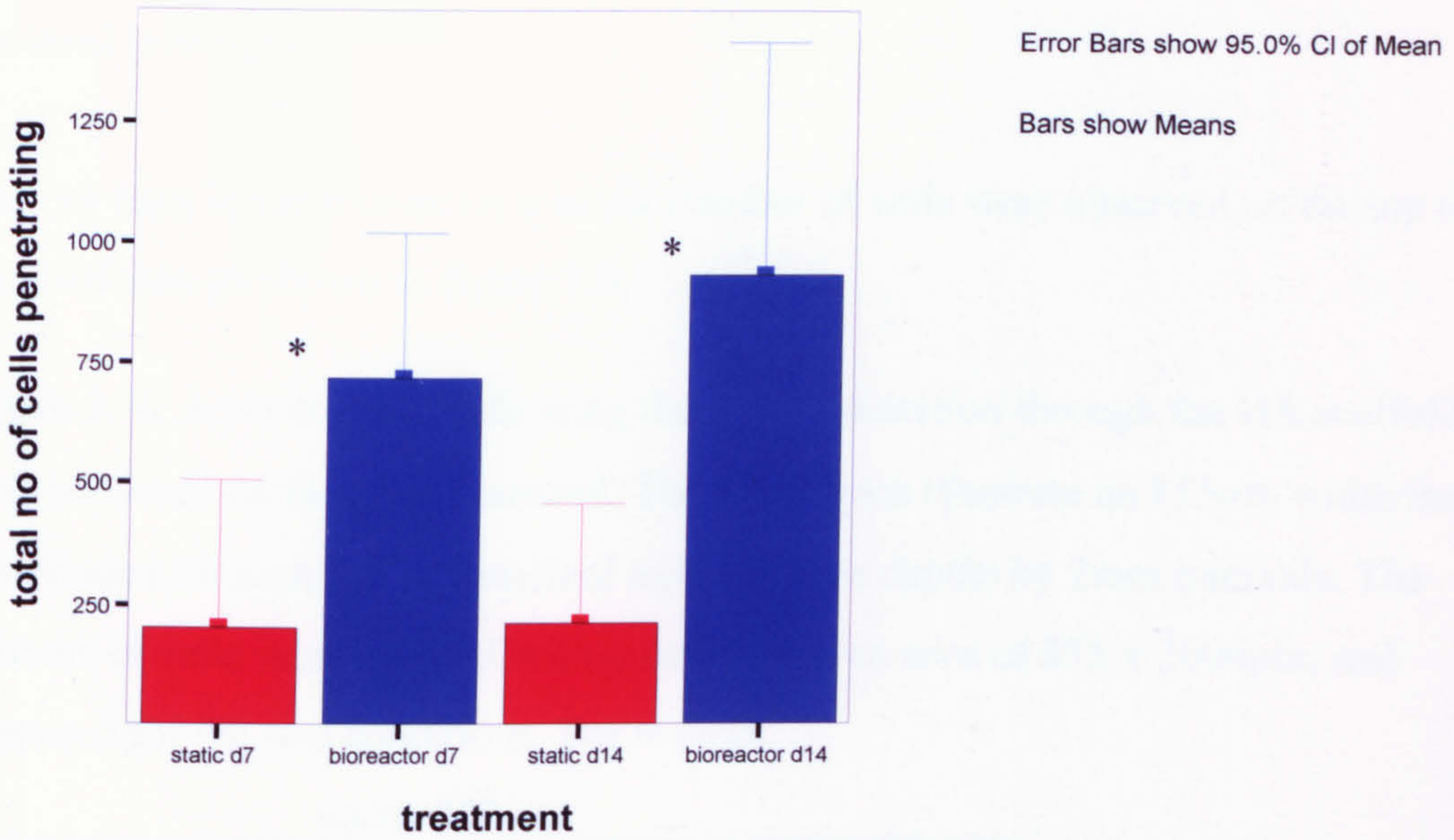


Figure 3. 3: Total number of cells on the cross sectional surface through HA scaffolds (counted within a 855μm width), comparing static with bioreactor culture after 7 and 14 days, * P-value <0.05 between static and bioreactor cell numbers.



4.3.1.2 Assessment of cell numbers at different depths through the HA

To further detail the penetration of cells through the HA scaffold, the cross sectional surface was divided into five layers, as described in the Method and illustrated in figure 3.4. The number of cells that had penetrated into each of these five layers through the cross-sectioned HA scaffold was compared between the two conditions (see figure 3.5).

The average number of cells that had penetrated into layers 2, 3 & 4 of the HA was significantly greater when cultured in the bioreactor than in static culture after 7 days ($P<0.05$), (see figure 3.5a). After 14 days in culture the cell penetration into layers 3 & 4 remained significantly greater for scaffolds in the bioreactor compared with the static system, with the addition of a significantly greater number of cells within the first layer in the bioreactor as compared to static culture ($P<0.05$), (see figure 3.5b).

Figure 3.6 show the HA scaffolds at 6mm depth (layer 3) for the two culture conditions after 7 days in culture, under SEM. In the bioreactor system, a network of

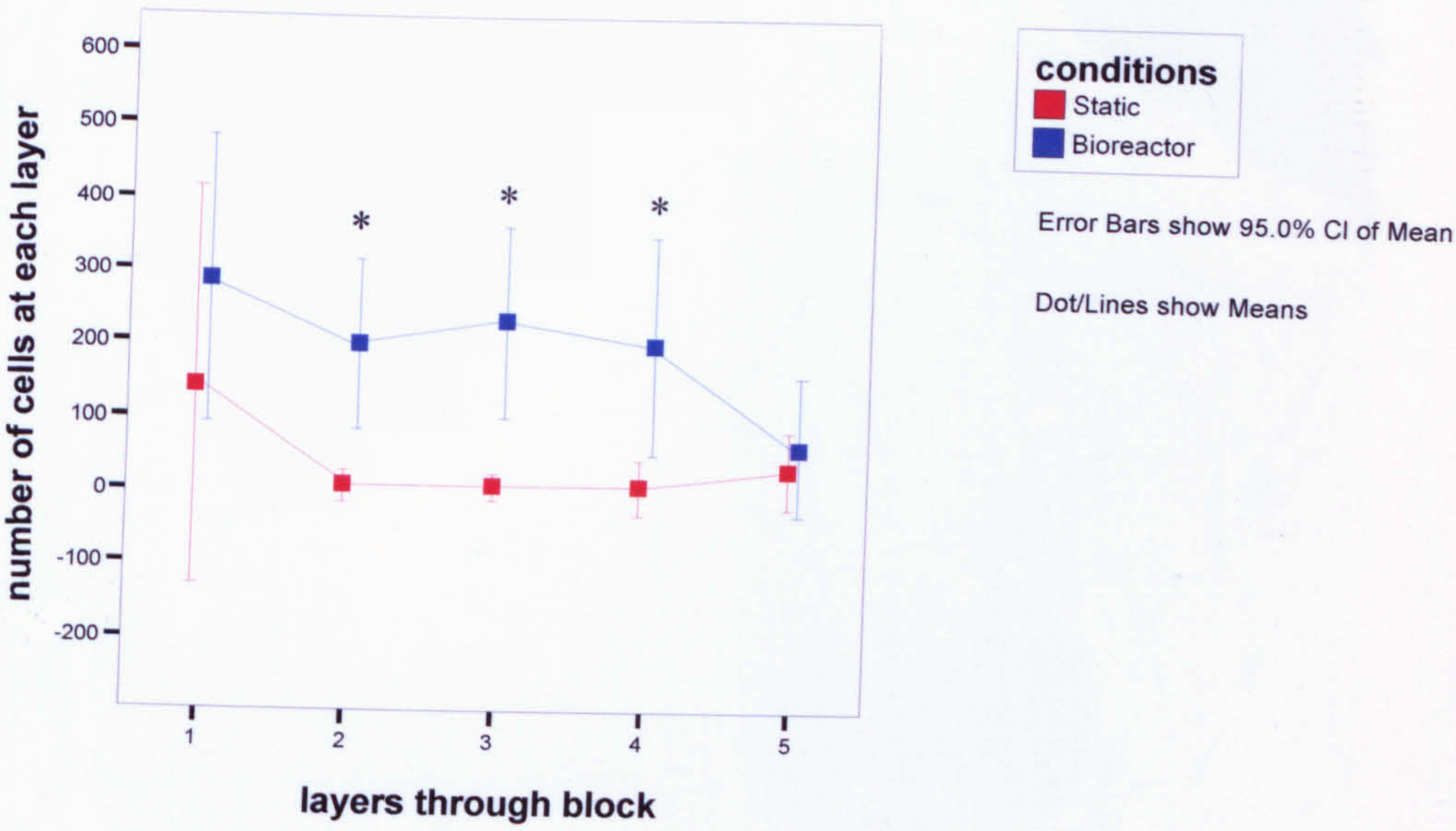
fibroblastic shaped cells formed on the HA at this depth, after 7 days. However in contrast, no cells were seen but there was evidence of cell debris at this depth in the porous HA, after 7 days in static culture (see figure 3.6b). By 14 days cells cultured in the bioreactor covered a large area of the scaffold (see figure 3.7a). Conversely, after the same time in static culture there were no cells and less evidence of cell debris at this level (see figure 3.7b).

After 14 days in the bioreactor a larger number of cells were observed on the top of the HA scaffolds, as shown in figure 3.8.

Figure 3. 4: A photograph indicating the how penetration through the HA scaffolds' cross-sectional surface was assessed. The black lines illustrate an 855μm width that was divided through the longitudinal axis into five depths by 2mm intervals. The numbers of cells were counted within each layer, an area of 855 x 2000μm, and compared for the two conditions. Bar = 1mm.

Figure 3. 5: Comparing the average number of cells counted within each layer of the cross section of the HA, for the two culture conditions, a) After 7 days in culture, b) After 14 days in culture, *P-value <0.05.

a)



b)

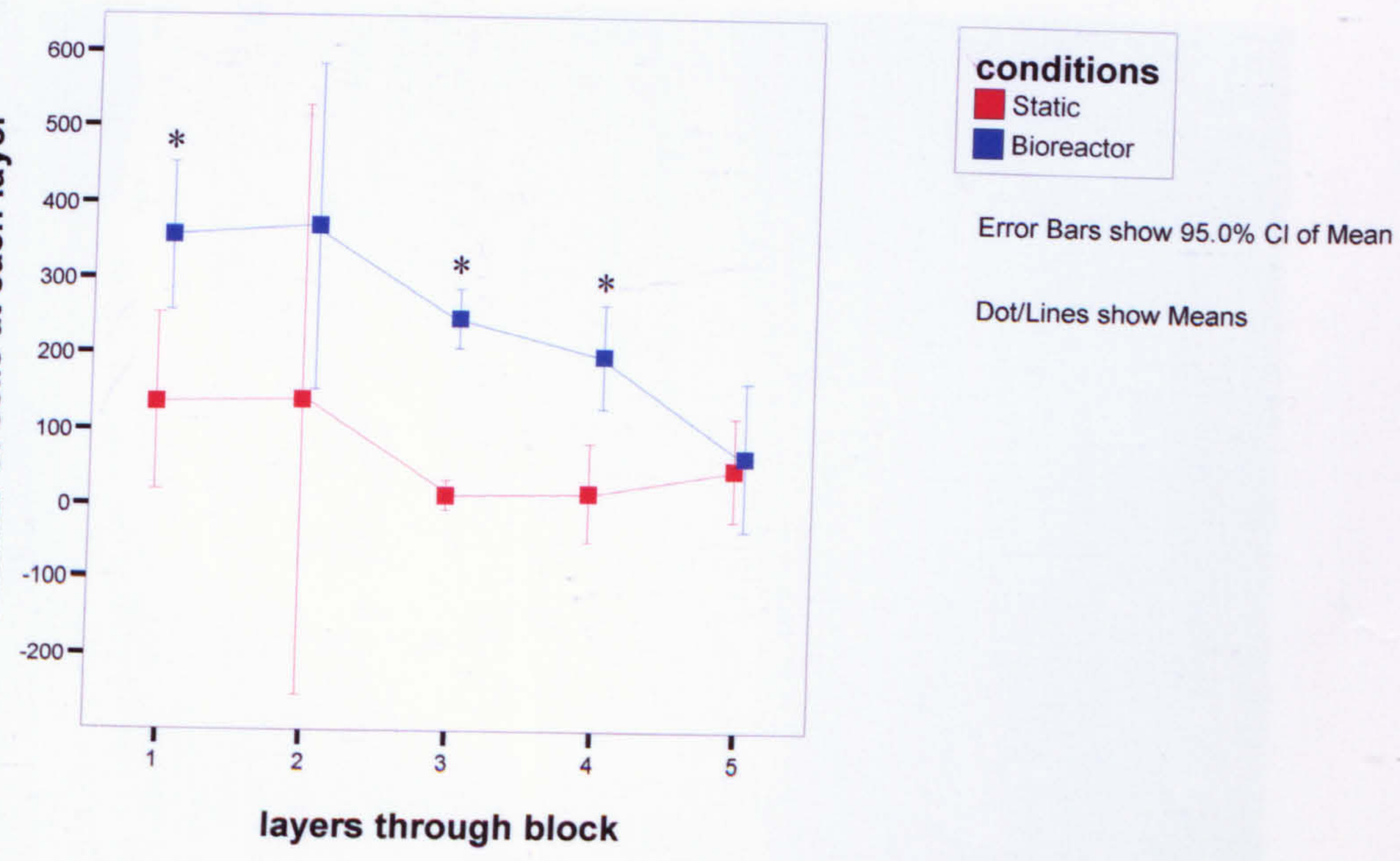
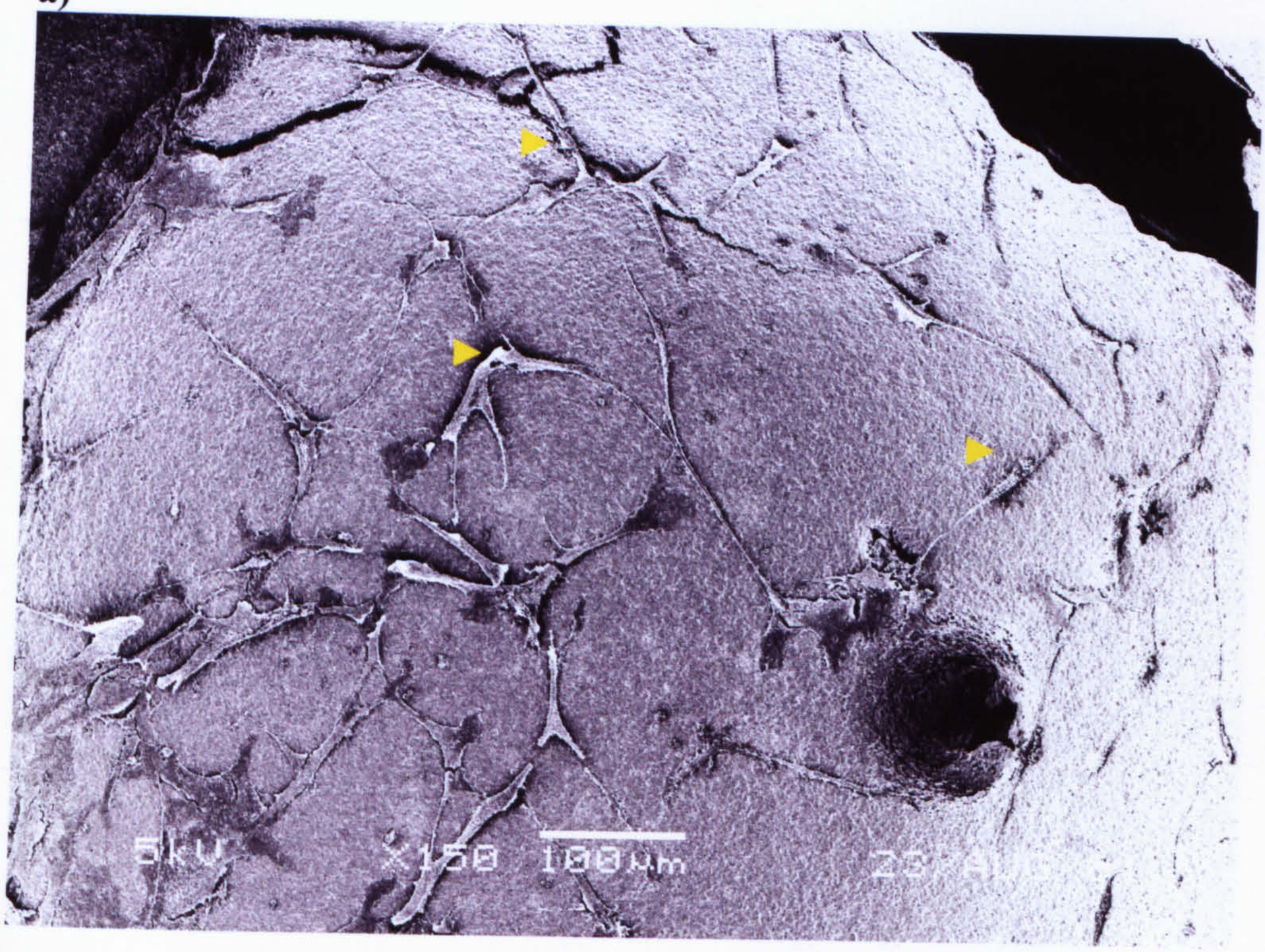


Figure 3. 6: SEM cross sectional pictures of static and bioreactor cultures to compare penetration at 6mm depth (layer 3) through HA scaffold after 7 days in a) the bioreactor and b) static culture. Yellow arrows indicate cells and pink arrows indicate cell debris. Bar = 100μm.

a)



b)

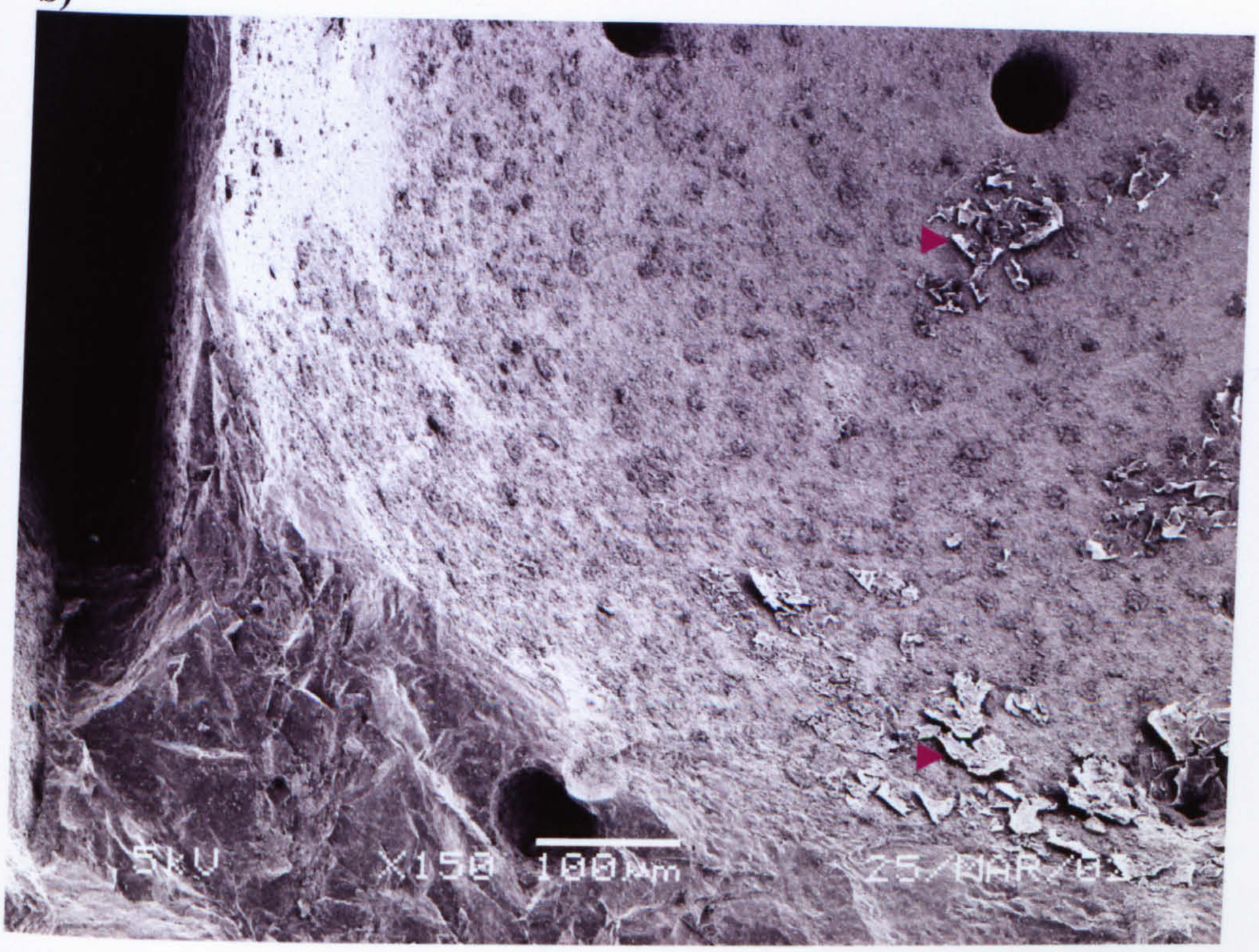
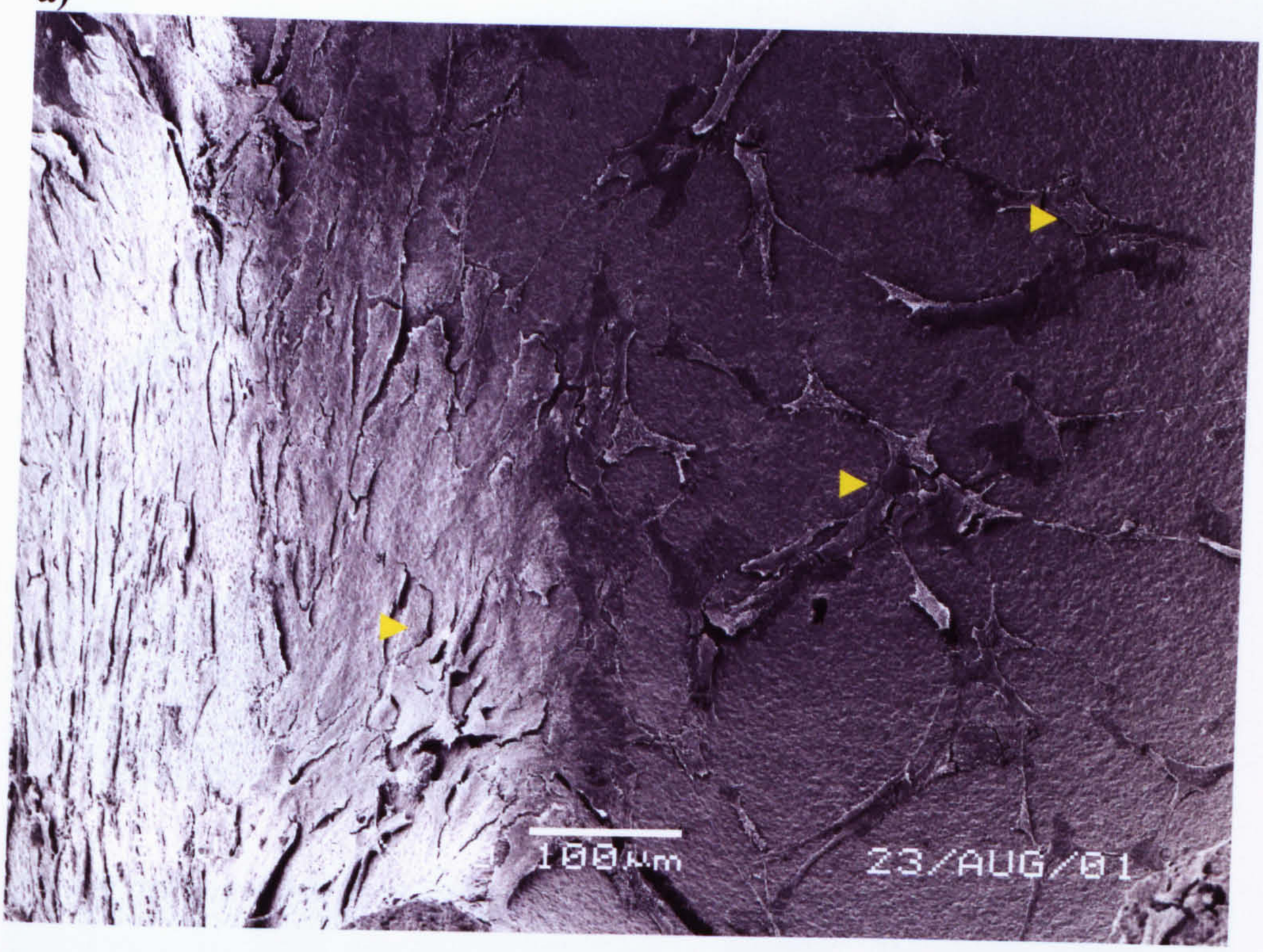


Figure 3. 7: SEM cross sectional pictures of static and bioreactor cultures to compare penetration at 6mm depth (layer 3) through HA scaffold after 14 days in a) the bioreactor and b) static culture. Yellow arrows indicate cells and pink arrows indicate cell debris. Bar = 100μm.

a)



b)

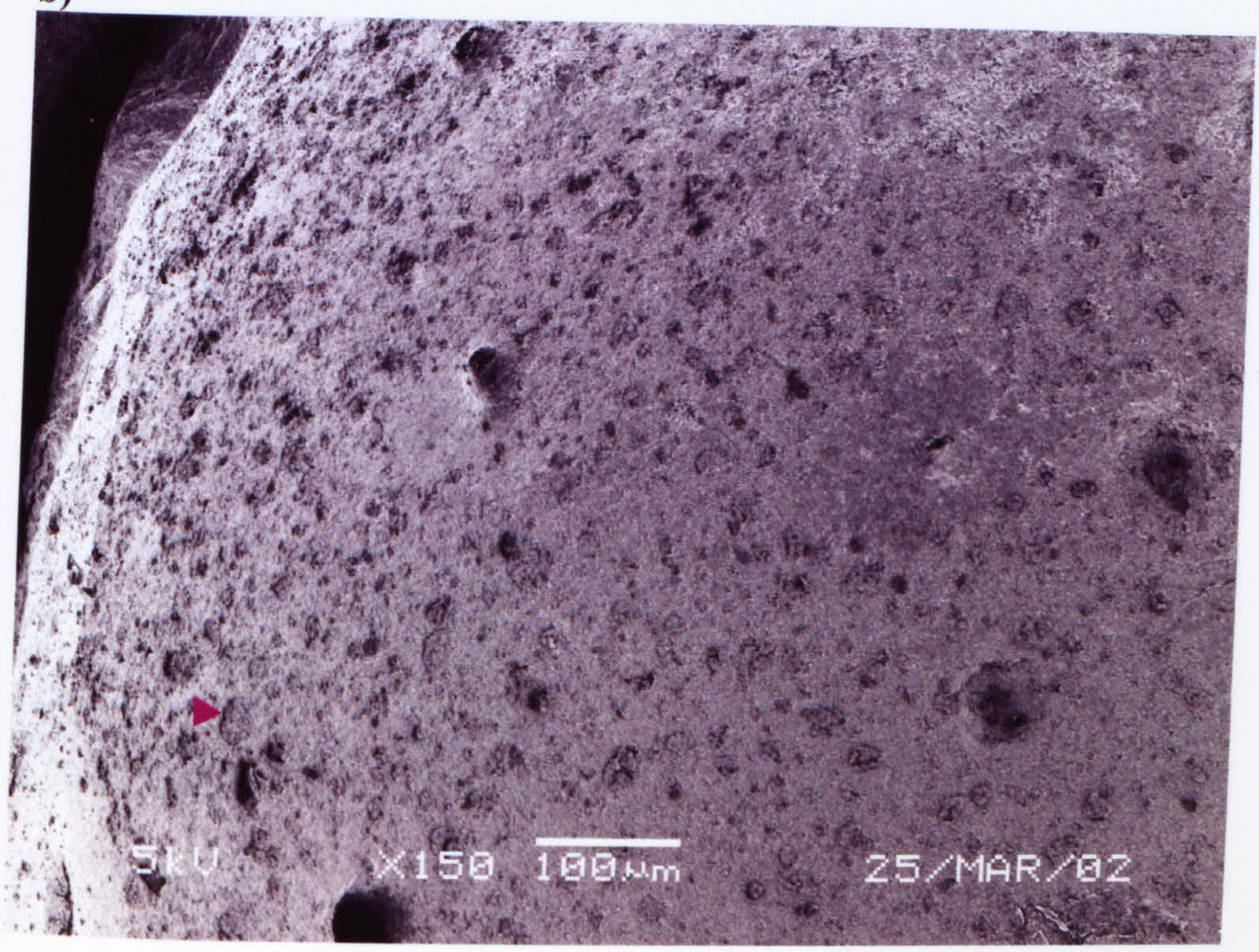
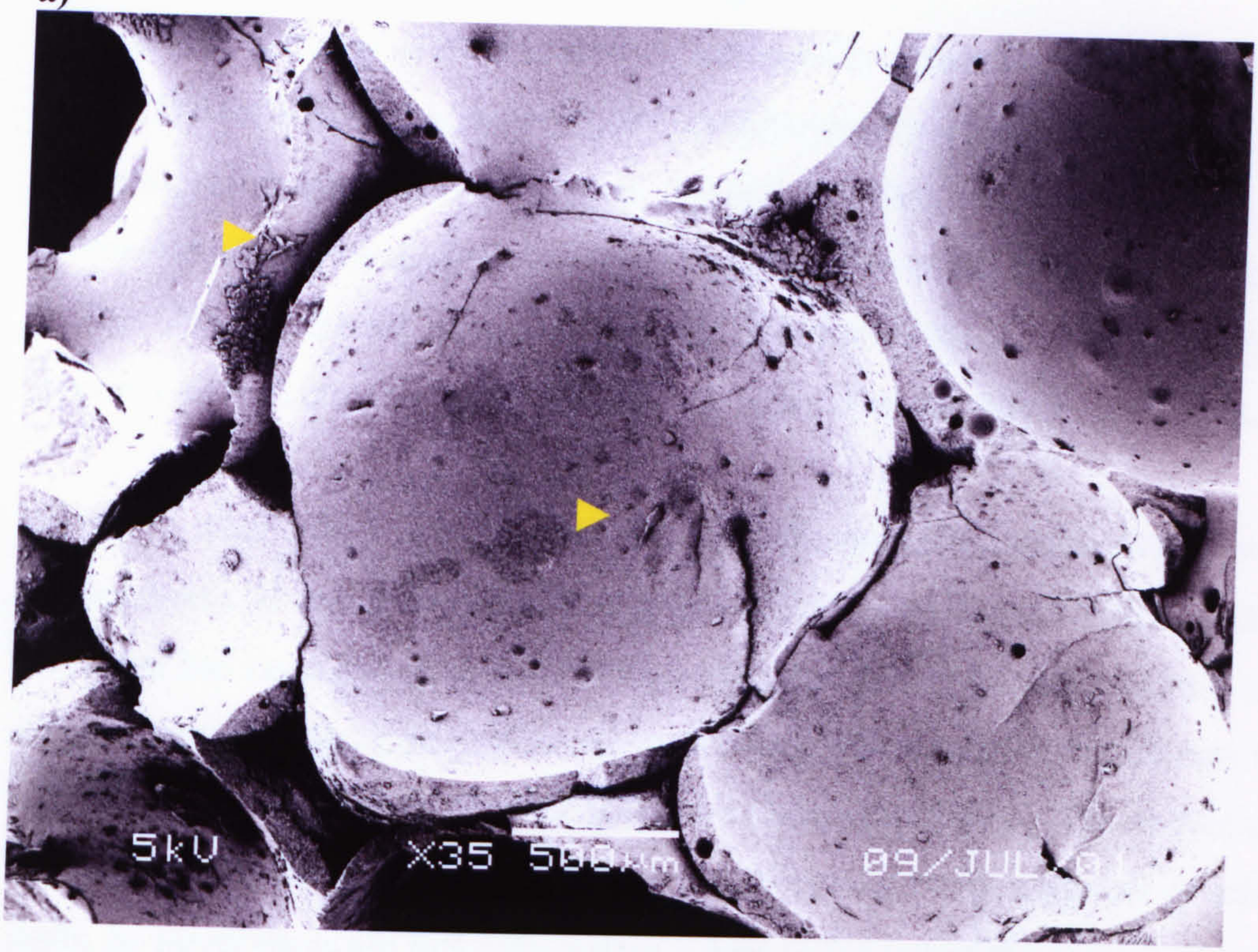
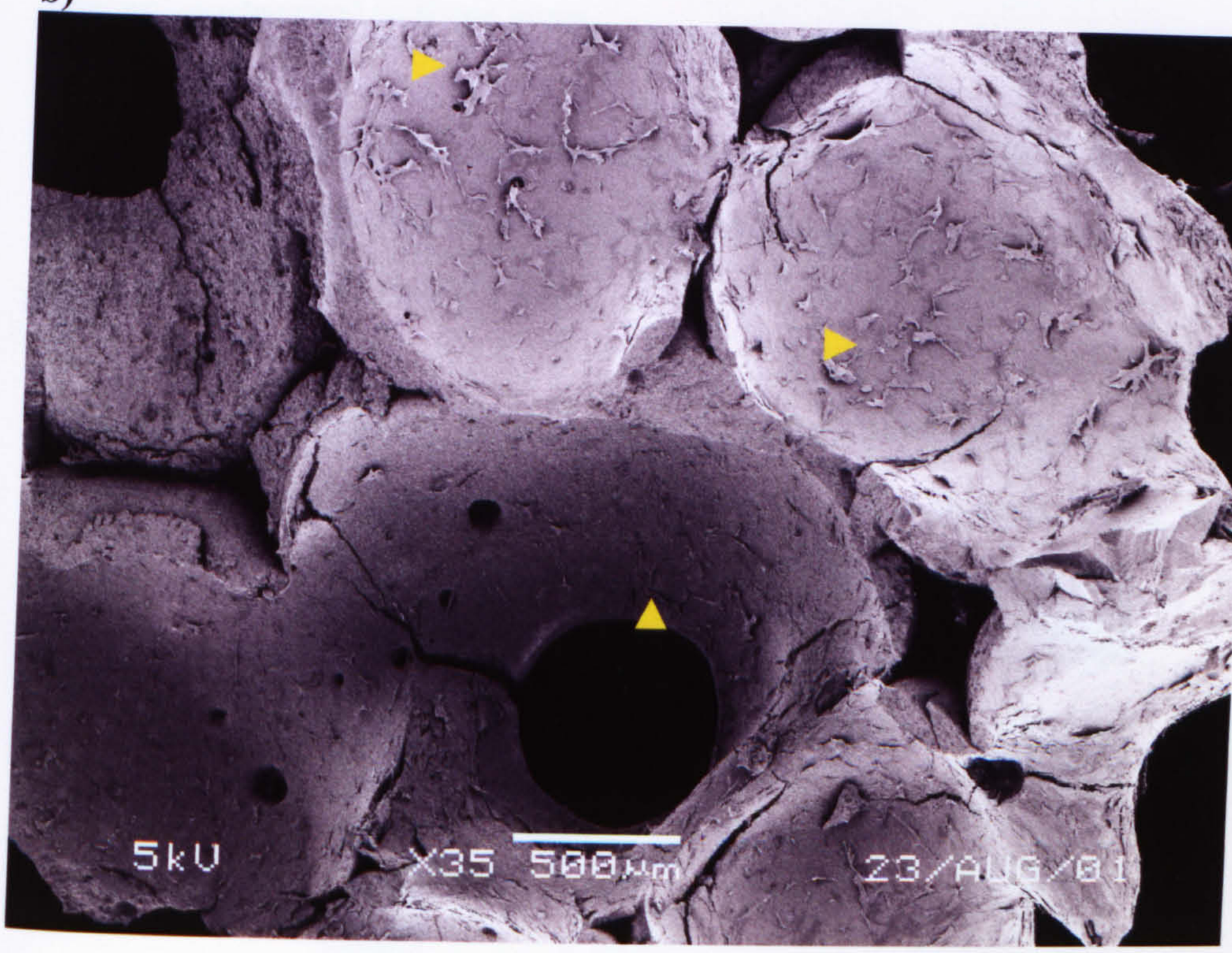


Figure 3. 8: Cells over the surface of the HA scaffold after 14 days cultured in a) static culture and b) the bioreactor, with yellow arrows indicating cells. Bar = 500μm.

a)



b)



4.3.3 Assessment of differentiation of MSCs in the bioreactor

4.3.3.1 Observations of SEM Morphology

The cellular differentiation was initially assessed by observing cell morphology under SEM. The cells were observed to form multiple processes with the HA surface, indicating attachment of the cells (see figure 3.9). After 7 days in culture in the bioreactor, a layer of spindle-shaped MSCs formed over the HA scaffold (see figure 3.10a). The cell density increased forming a mesh over the HA, and by 14 days the majority of the cells changed becoming cuboidal in shape (see figure 3.11a).

However, after 7 days in static culture fewer cells were observed and there was evidence of cell debris on some parts of the scaffold (see figure 3.10b). By day 14, cells that had been grown in static culture also changed in morphology into squarer shaped cells, but the degree of coverage of the scaffolds was much less than for those cultured in the bioreactor. An example of this is seen in figure 3.11b, where a ridge of HA scaffold on the right of the picture is totally covered with a mosaic of cuboidal cells, however there are few cells within a pore to the left of the picture and cell debris was again observed in these areas.

The cell morphology was also studied at higher magnification. After 7 days in culture in the bioreactor the cells were typically spindle in shape, however by 14 days the cells became squarer (see figure 3.12).

Figure 3. 9: SEM picture showing cell processes attaching cells to the HA, with part of a cell shown by a blue arrow and processes shown with pink arrows. Bar = 5μm.

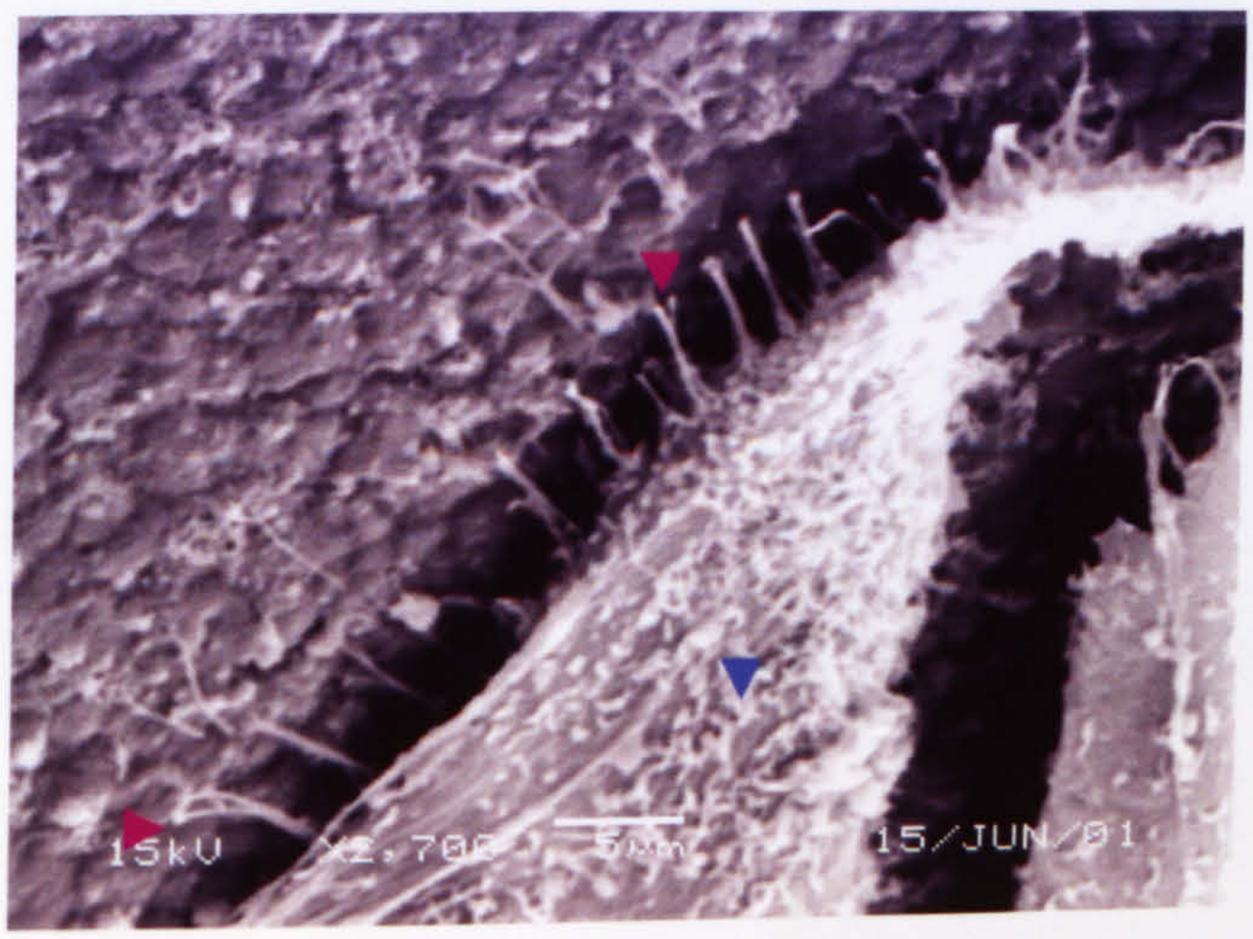
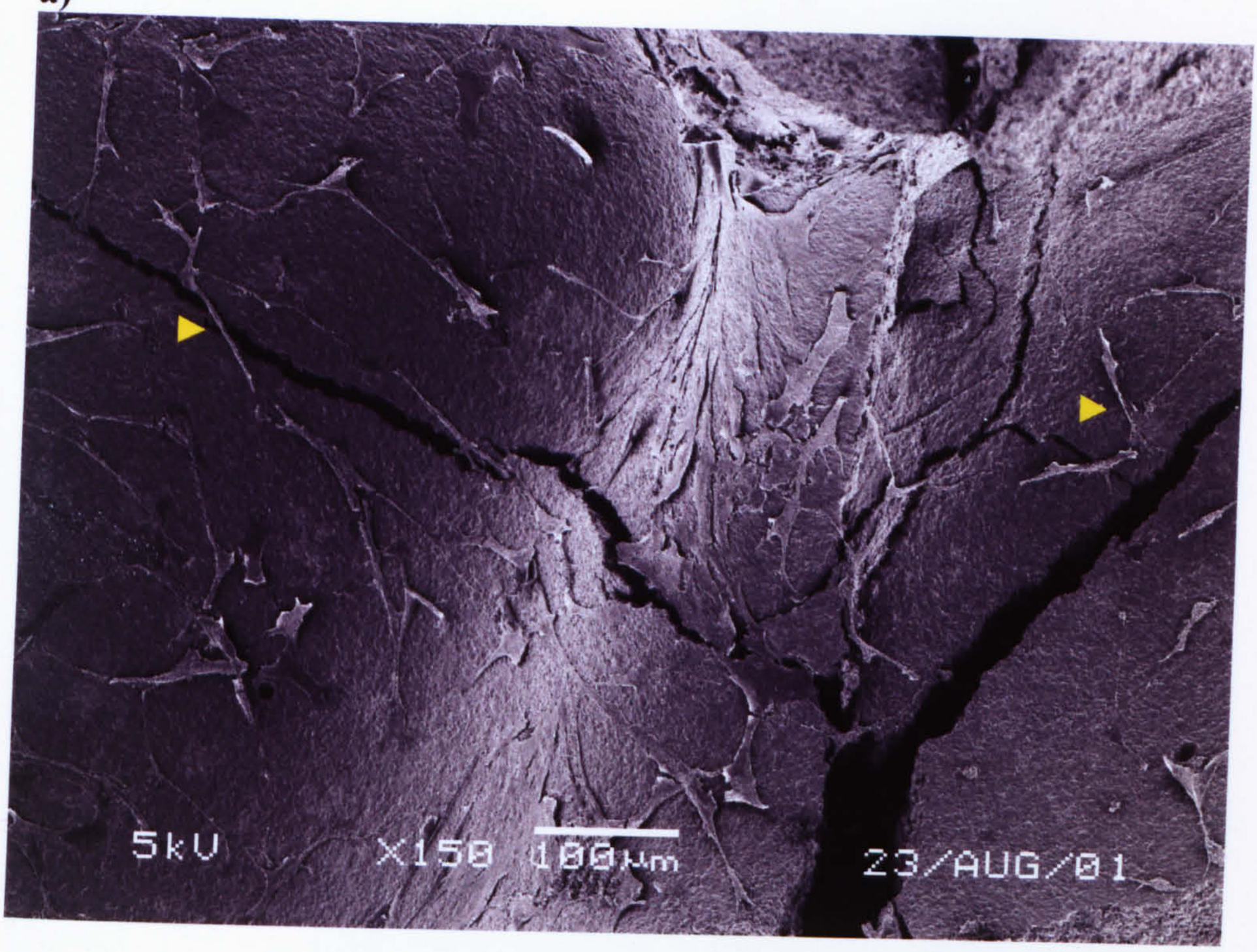


Figure 3. 10: SEM of MSCs grown on HA in each condition showing the relative cell density and morphology after 7 days in a) the bioreactor and b) static culture. Yellow arrows indicate spindle-shaped cells and pink arrows cell debris, bars = 100μm.

a)



b)

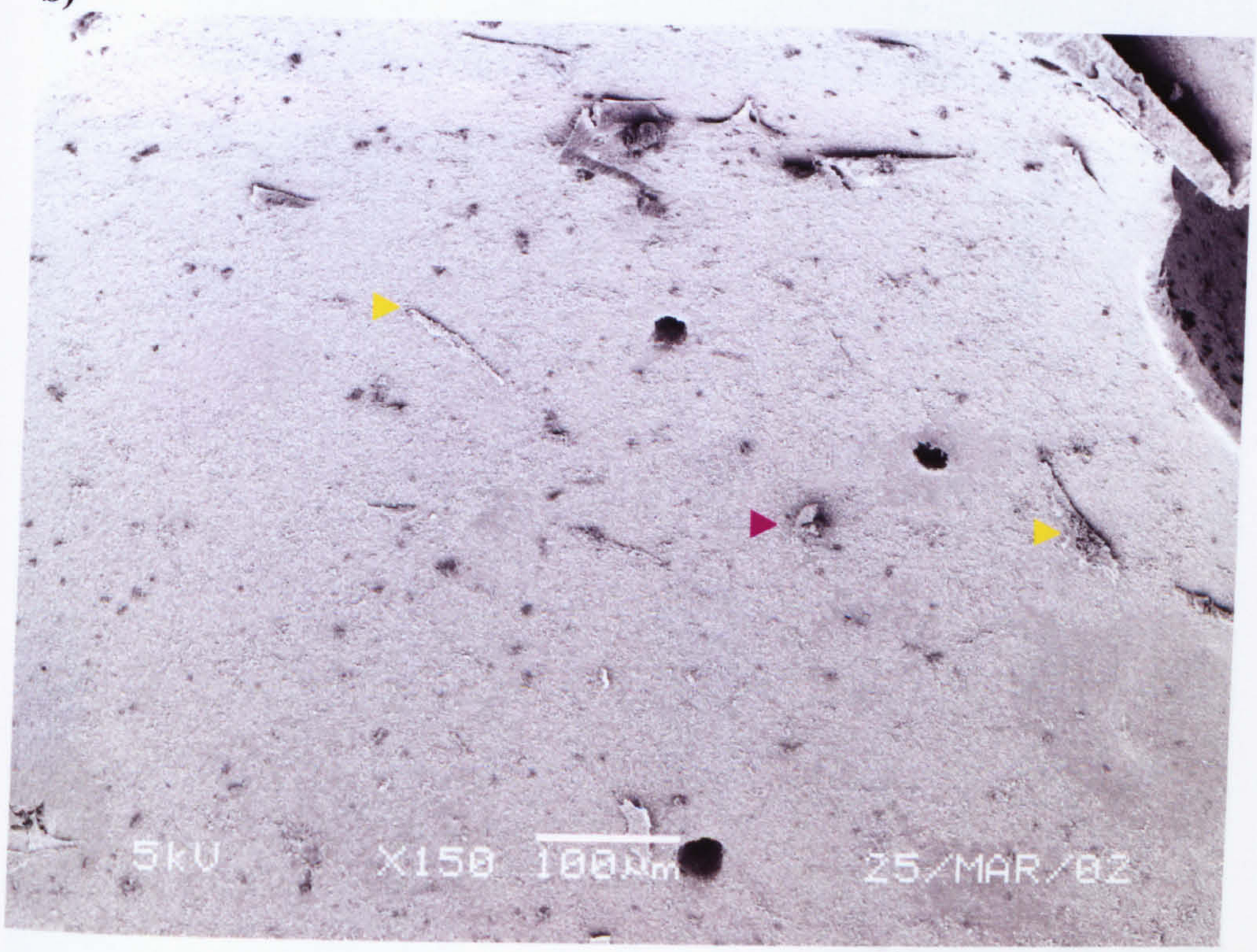
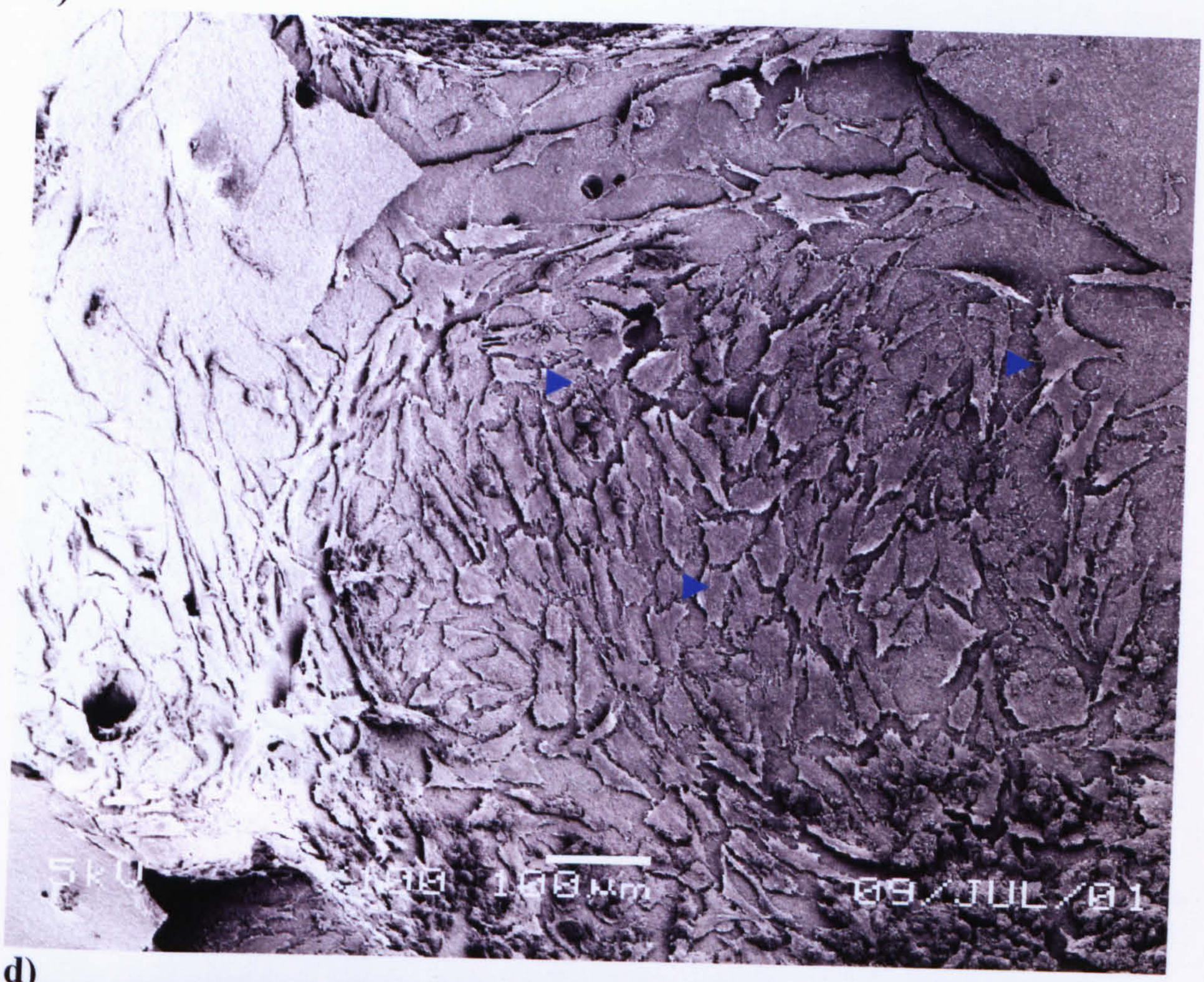


Figure 3. 11: SEM of MSCs grown on HA in each condition showing the relative cell density and morphology after 14 days in a) the bioreactor (bar = 100μm) and b) static culture (bar = 50μm). Blue arrows show cuboidal-shaped cells and pink arrows indicate evidence of cell debris.

c)



d)

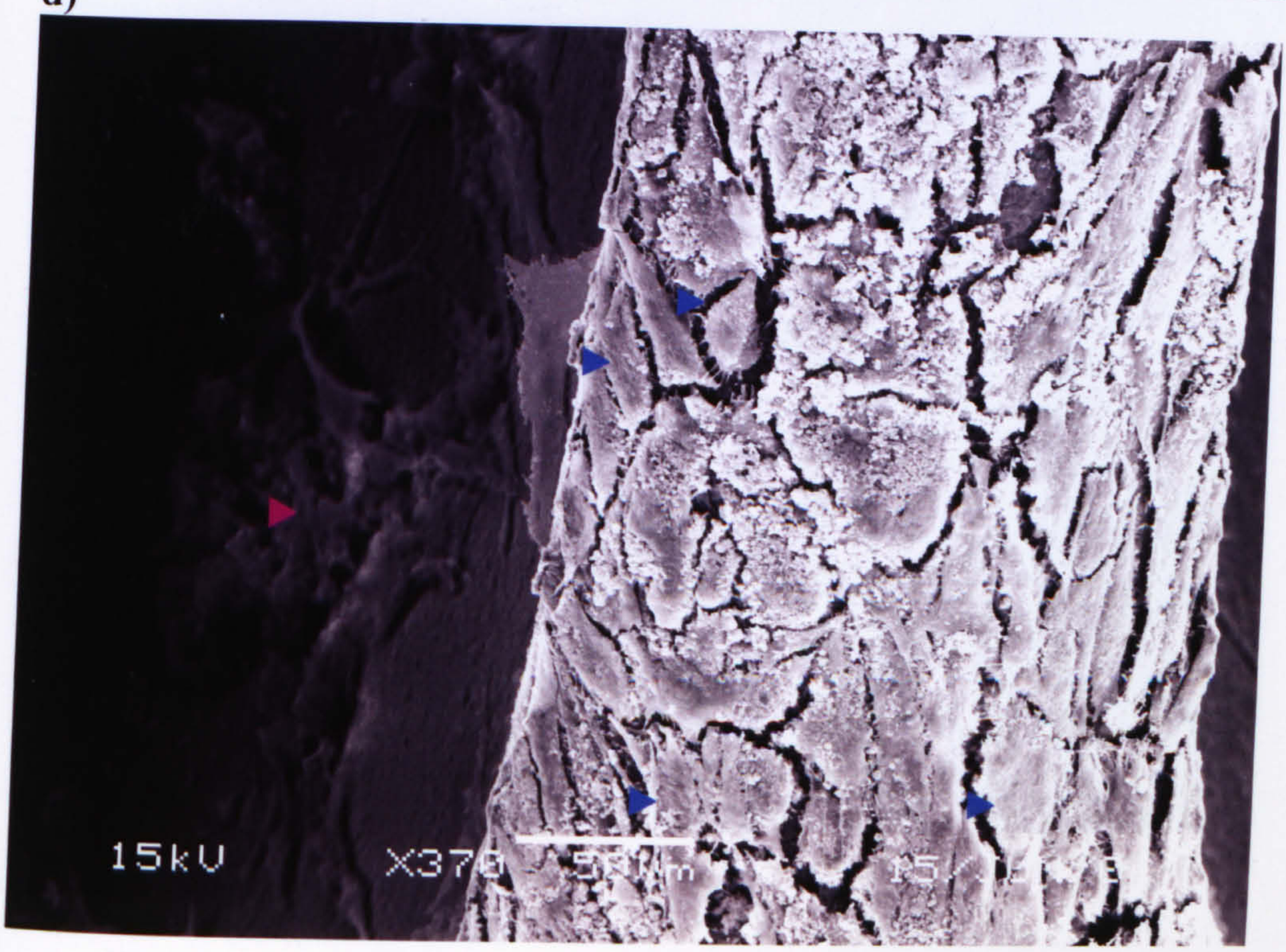
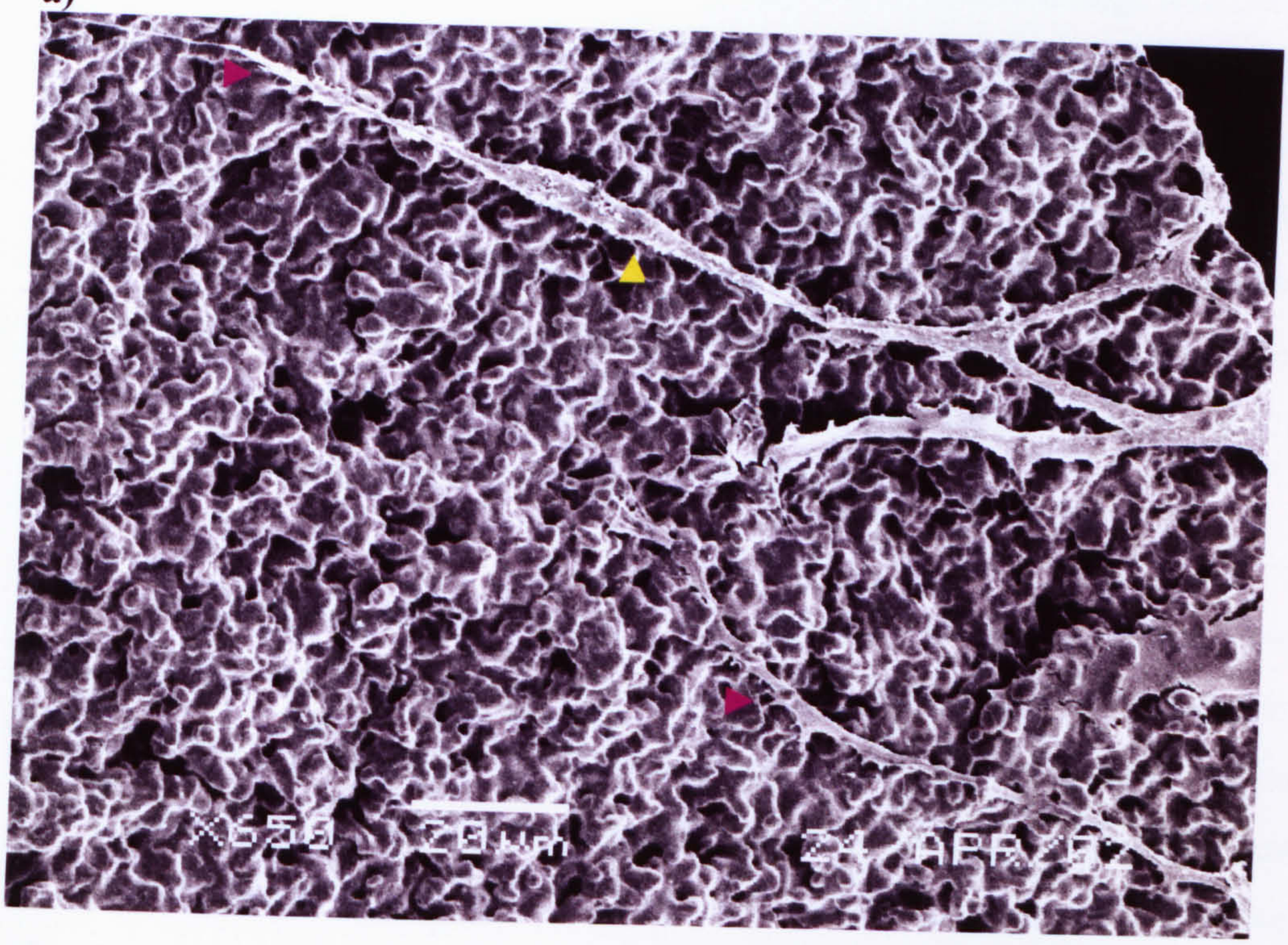
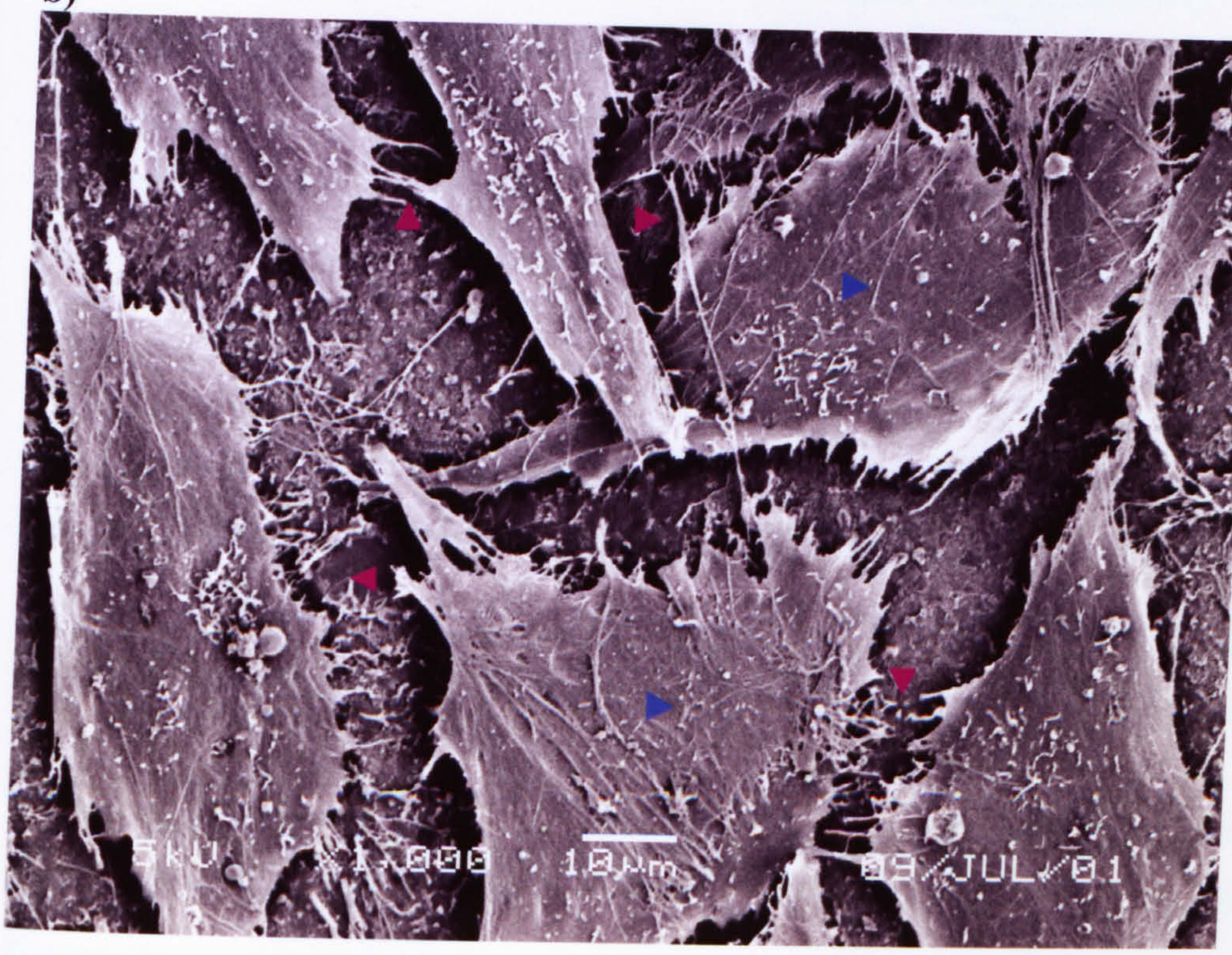


Figure 3. 12: Morphology of cells following culture in the bioreactor after a) 7 days showing spindle-shaped cells, yellow arrows, bar = 20μm and b) 14 days showing cuboidal cells, blue arrows, bar = 10μm. Cell processes shown with pink arrows.

a)



b)



4.3.3.2 Observations of TEM results

Initially the HA scaffolds were observed under light microscopy to find the density and distribution of cells on the scaffolds. Figure 3.13 shows a large number of cells filling the pores of the HA scaffolds following culture in the bioreactor for 28 days. At higher magnification individual cells can be seen spanning across gaps in the HA (see figure 3.13b). However following the same culture time in static culture, a dramatic difference in cell numbers on the HA was observed (see figure 3.14). At low magnification no dense cellular areas were seen, although at higher magnification some cells were noted around the HA. For each scaffold areas of higher cell density were identified and further examined under TEM.

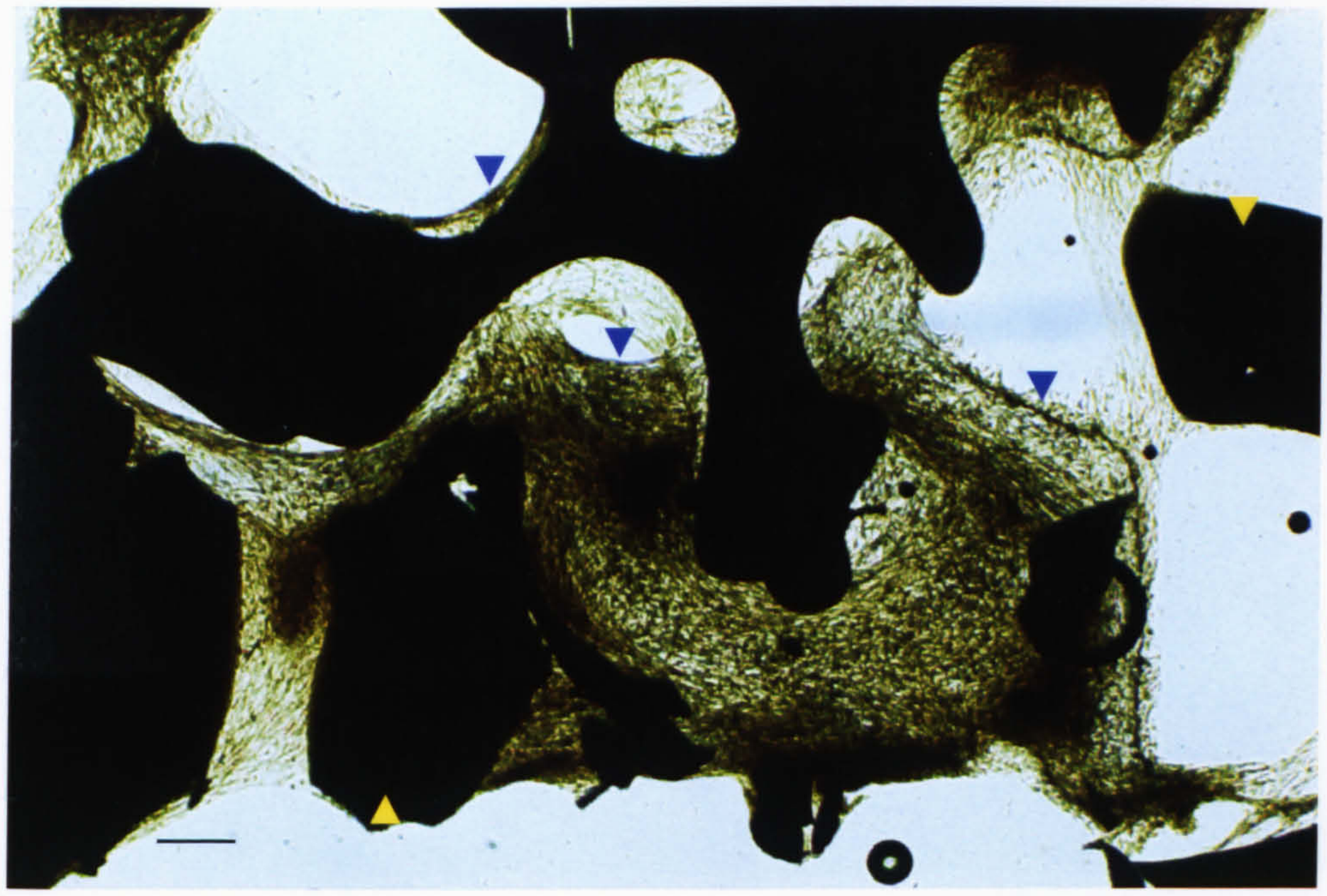
Cells were shown attaching to the HA scaffold when cultured in the bioreactor under TEM (see figure 3.15). After 14 days of culture in the bioreactor cells with multiple cell processes and containing rough endoplasmic reticulum were observed (see figure 3.16). After 28 days in the bioreactor, cells were also noted to contain lysosomal bodies and vesicles (see figure 3.17); others contained large amounts of intracellular glycogen and rough endoplasmic reticulum (see figure 3.18). Additionally, intracellular lipid droplets were observed in some of the cells that had been cultured in the bioreactor (see figure 3.19a and 3.20a). Furthermore, after 28 days in the bioreactor extracellular matrix of collagen fibres were produced (see figure 3.19 & 3.20). Periodicity of the collagen fibres was seen, although this was faint (see figure 3.19b & c).

Adjacent to some areas of organised collagen matrix, mineralised crystalline deposits were observed (see figure 3.21a). The composition of these areas was found to contain calcium and phosphate on energy dispensive x-ray analysis (EDAX) probing, with a ratio of 1.8 (see figure 3.21b & c).

In static culture however, after 14 days in culture cells were too infrequent to be seen under high magnification of TEM. At 28 days cells were seen, containing intracellular organelles (see figure 3.22) and some evidence of collagen formation on one of the scaffolds (see figure 3.23), but this was uncommon compared to those cultured in the bioreactor.

Figure 3. 13: HA scaffold (yellow arrows) following 28 days in the bioreactor, viewed under light microscopy showing large numbers of cells filling the pores (stained brown by osmium, blue arrows) a) bar = 500μm b) bar = 100μm.

a)



b)

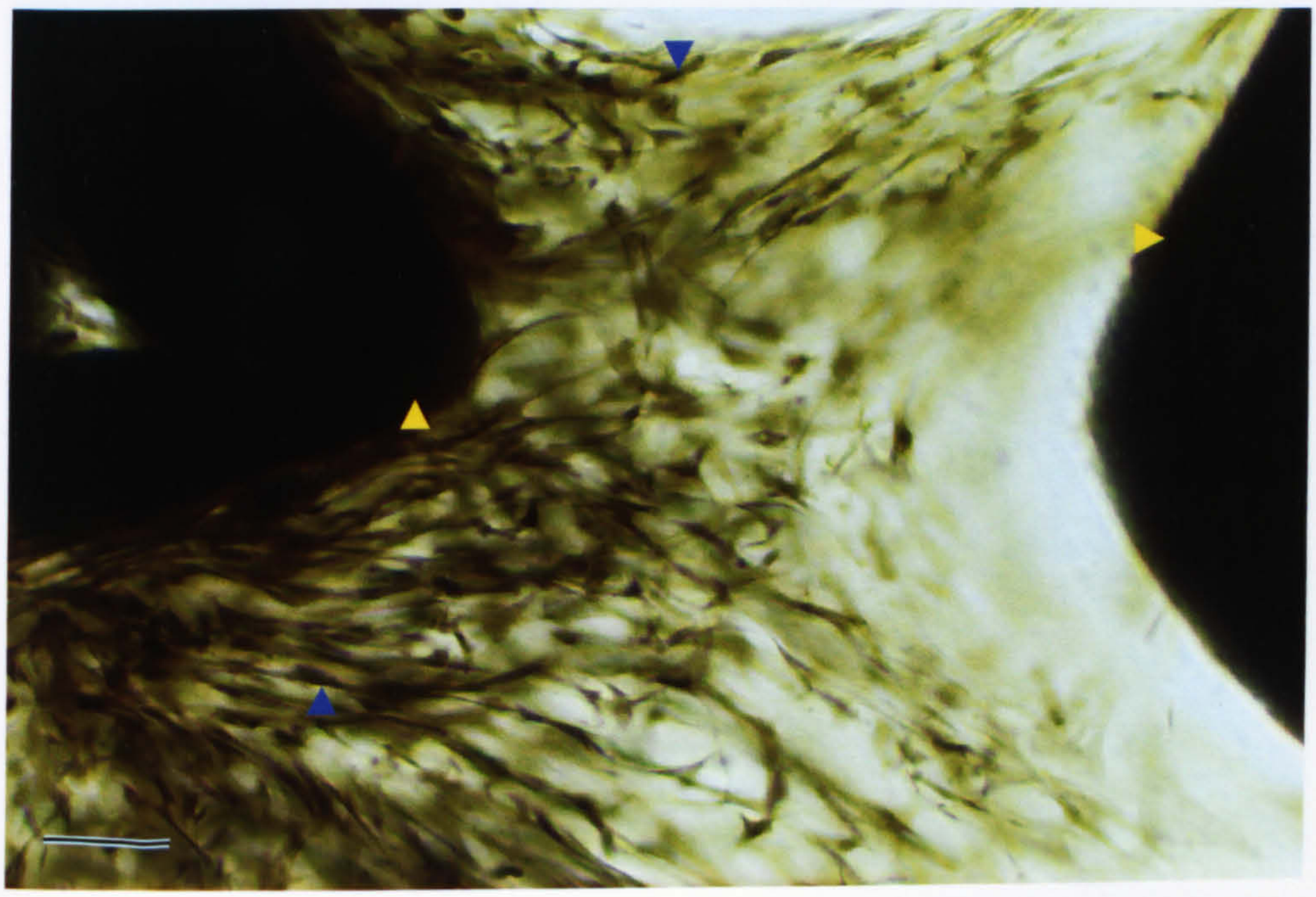
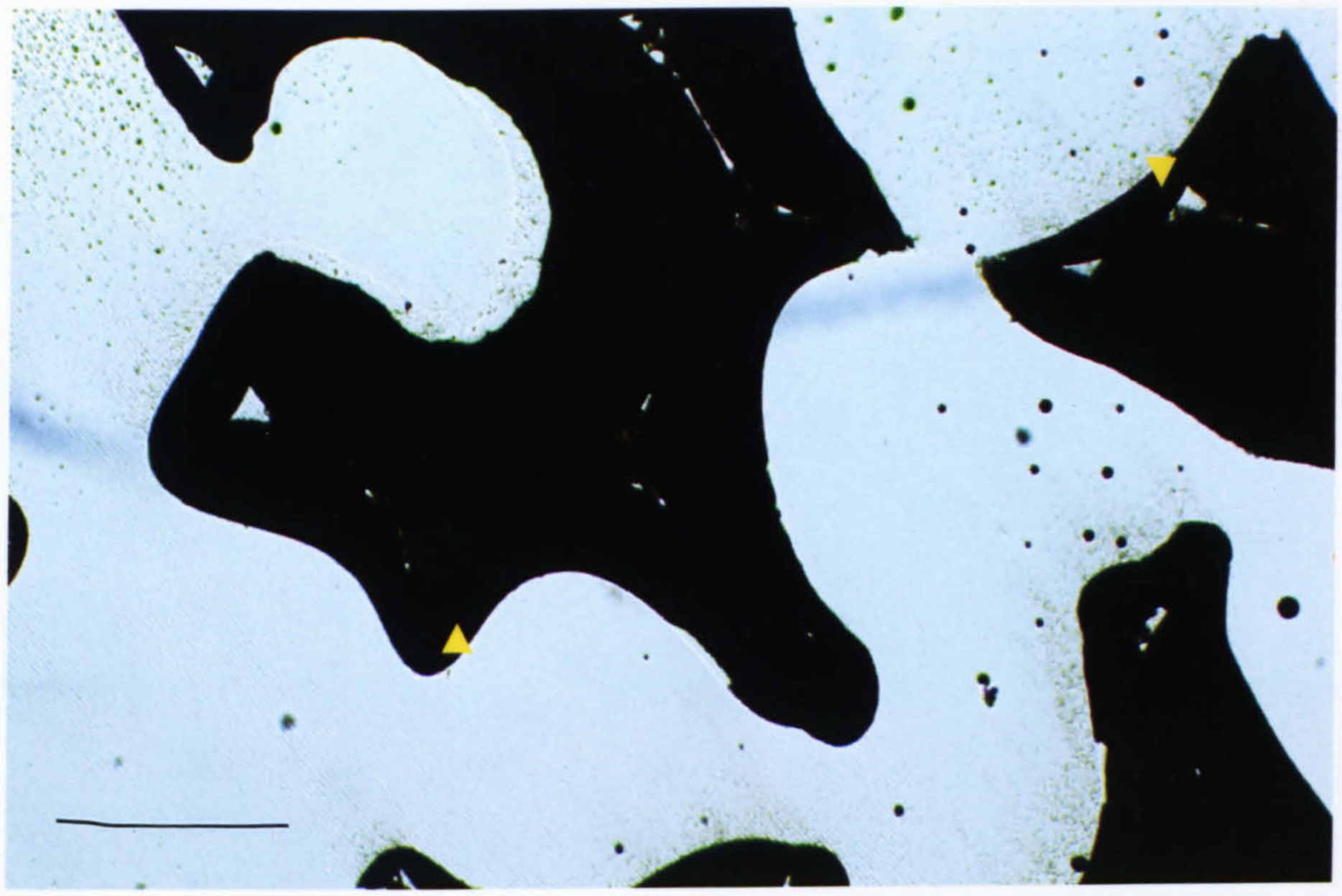


Figure 3. 14: HA scaffold (yellow arrows) following 28 days in static culture, viewed under light microscopy showing fewer cells (stained brown by osmium, blue arrows) a) bar = 500μm b) bar = 100μm.

a)



b)

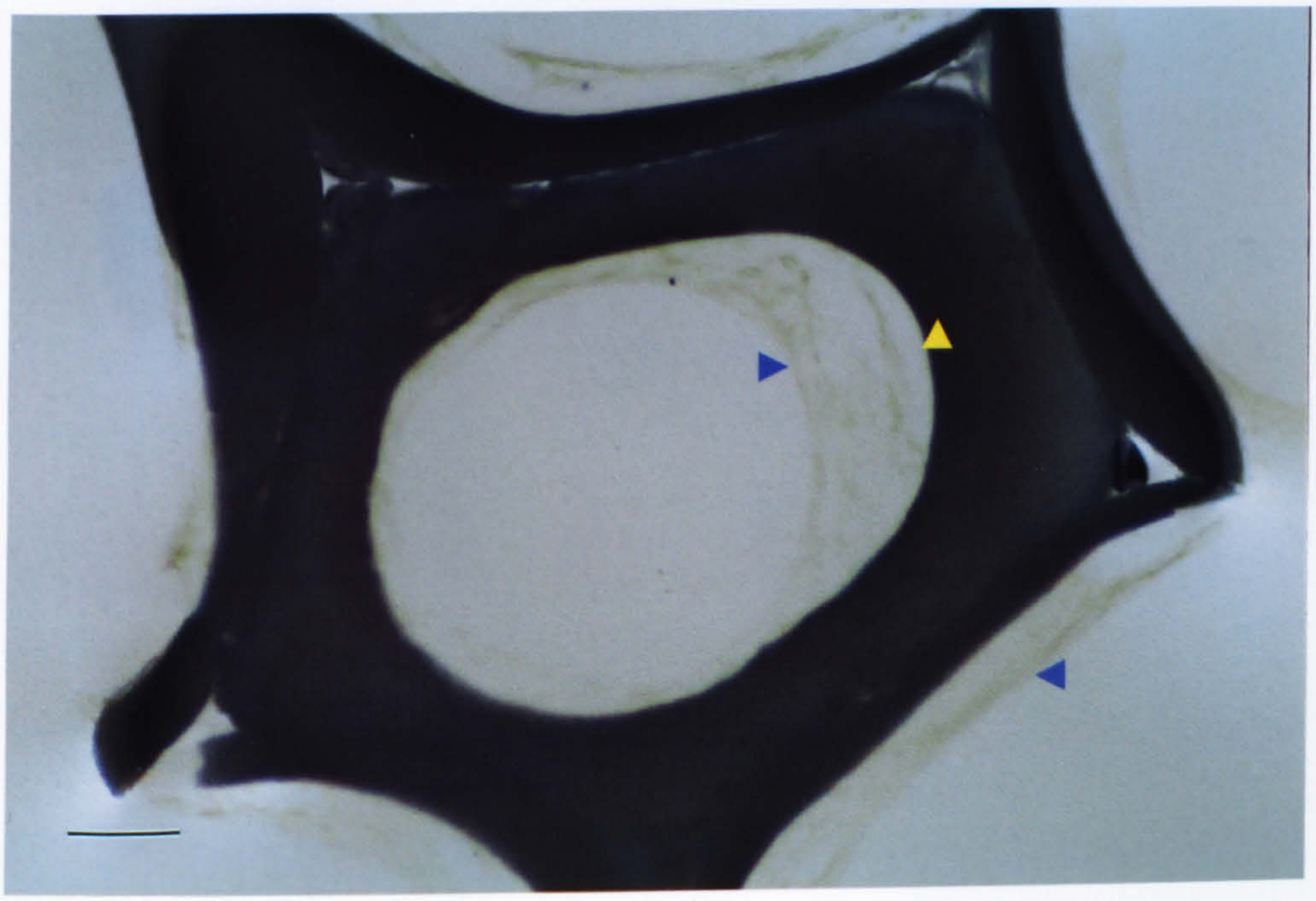


Figure 3. 15: TEM of cells around HA scaffold following culture in the bioreactor showing a cell with nucleus (N), surrounding the HA (H). Bar = 1.5μm.

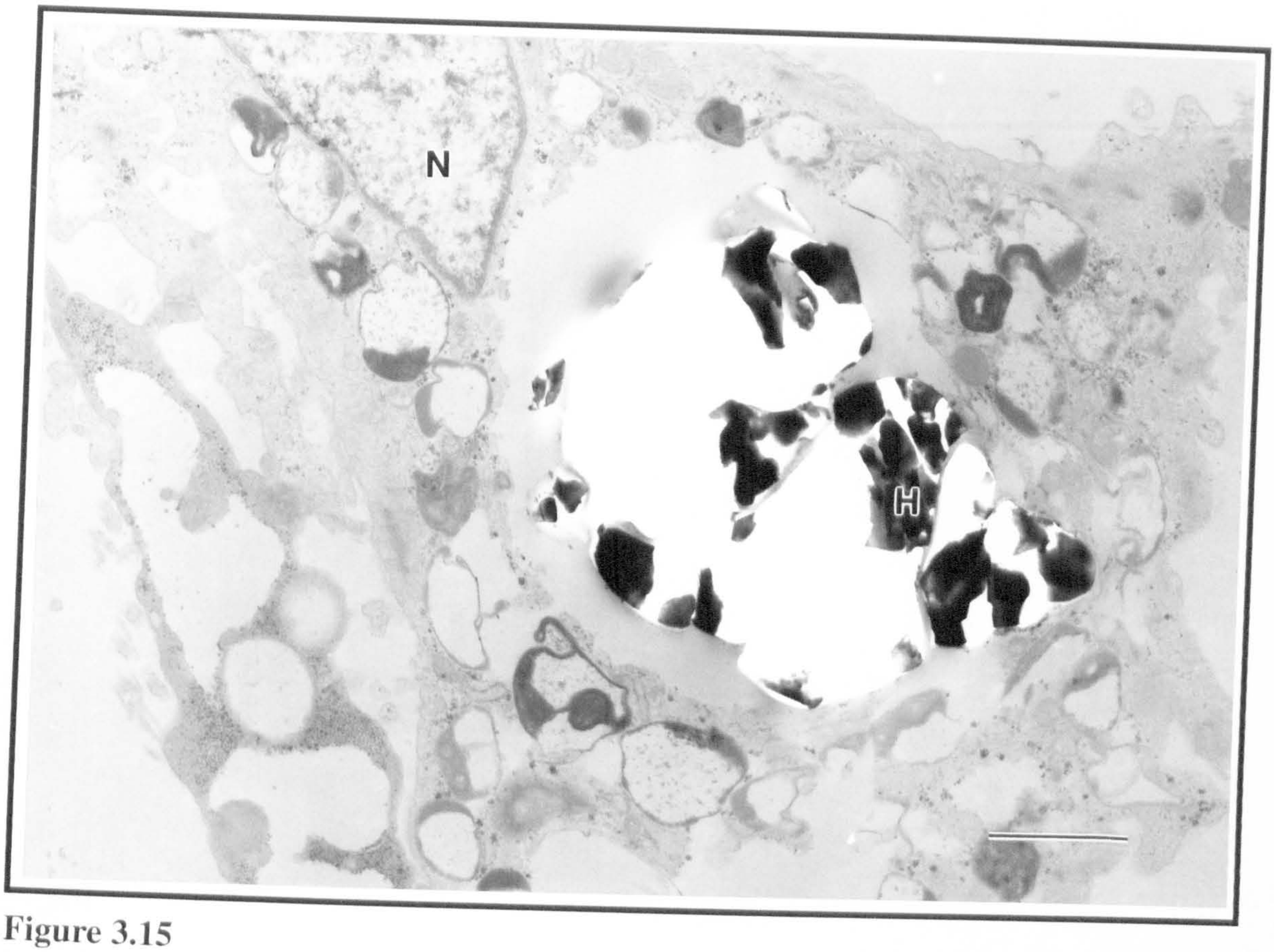


Figure 3.15

Figure 3. 16a: Cells with nuclei (N) containing nucleoli and endoplasmic reticulum (arrowheads) after 14 days culture in the bioreactor. Cell processes (arrows) are in abundance. Bar = 3μm.

Figure 3. 16b: Higher magnification of cells after 14 days in the bioreactor showing nucleus (N), various stages of lysosomal body formation (L) and rough endoplasmic reticulum (arrowheads). Bar = 1.5μm.

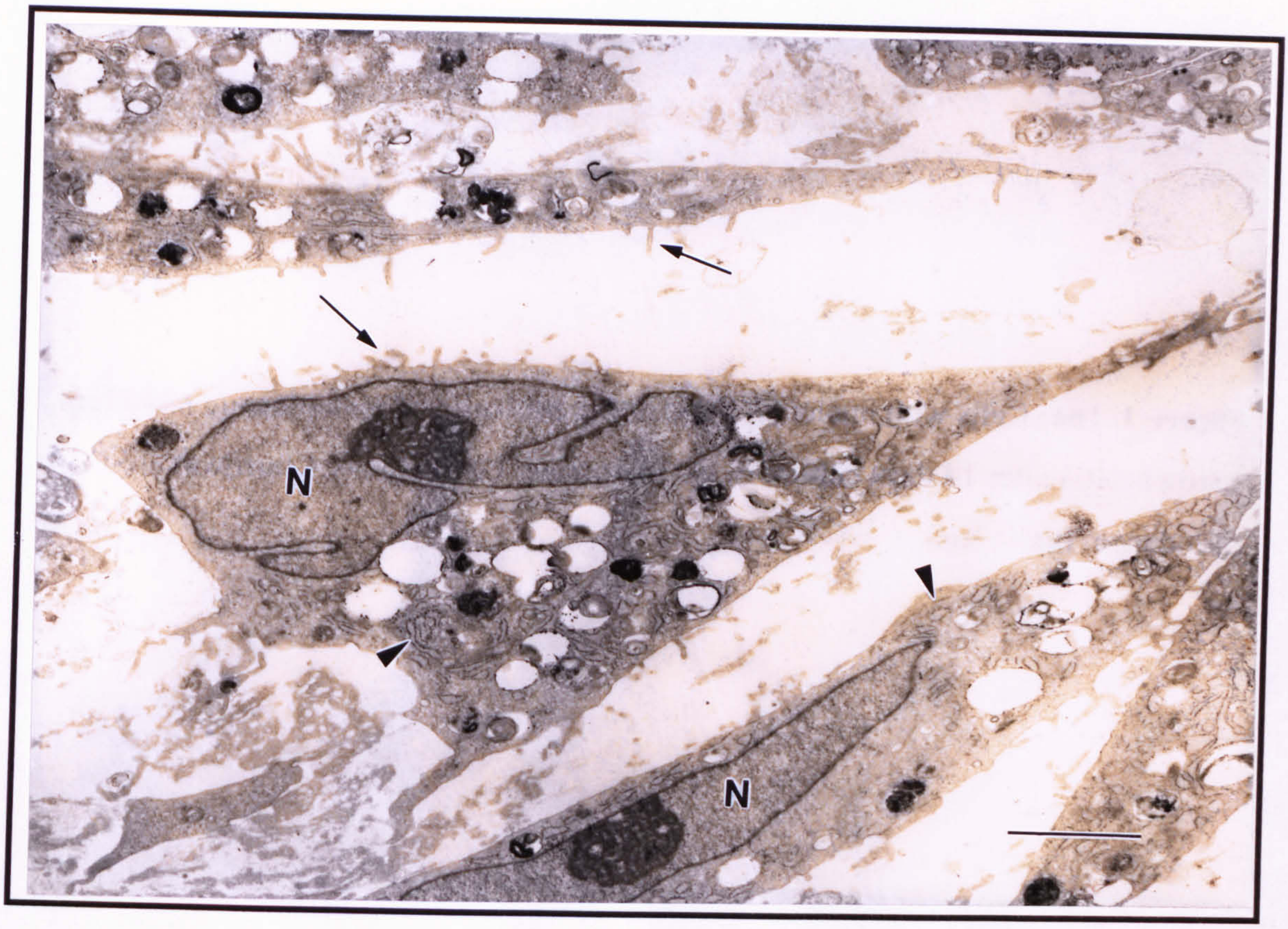


Figure 3.16a

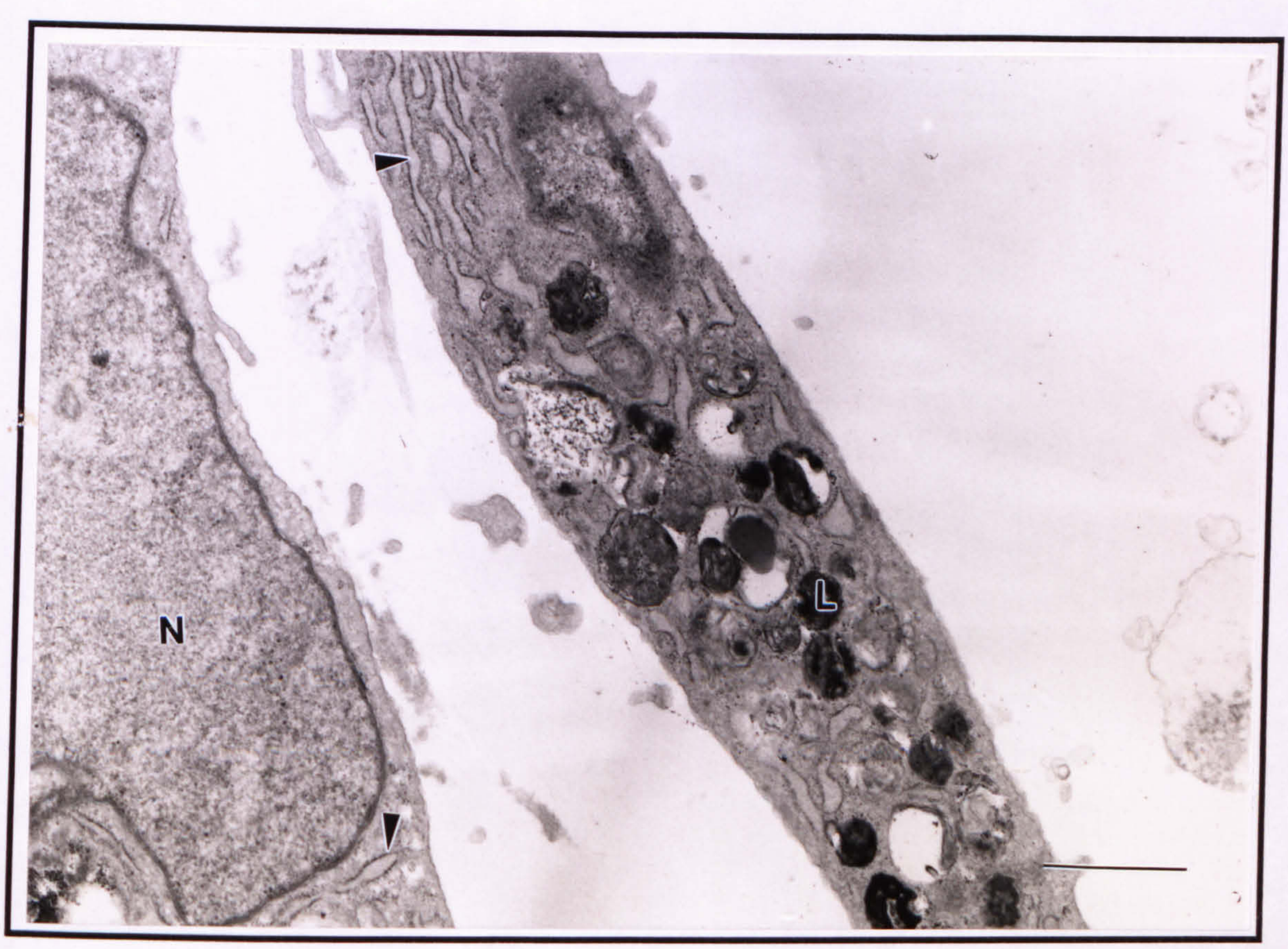


Figure 3.16b

Figure 3. 17: After 28 days in bioreactor culture, cellular ultrastructure additionally shows lysosomal bodies (arrows), rough endoplasmic reticulum (arrowheads) and intracellular glycogen (G). Cell processes are again evident. Bar = 3μm.

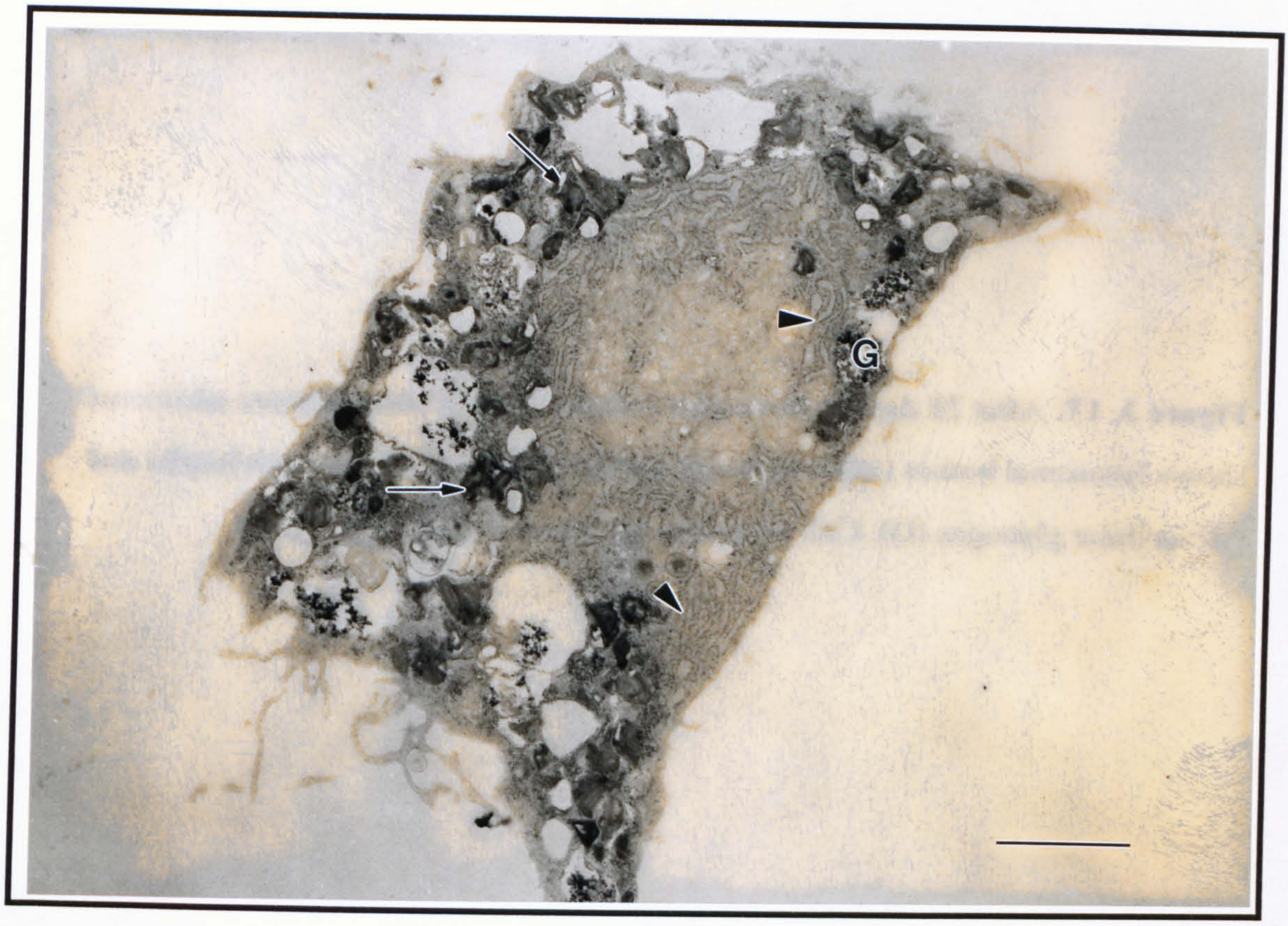


Figure 3.17

Figure 3. 18a: After 28 days in bioreactor culture, a cell containing much endoplasmic reticulum (arrows) and glycogen (G). Bar = 3μm.

Figure 3. 18b: A higher magnification of the same cell, highlighting the rough endoplasmic reticulum (arrow) and glycogen (G). Bar = 0.6μm.

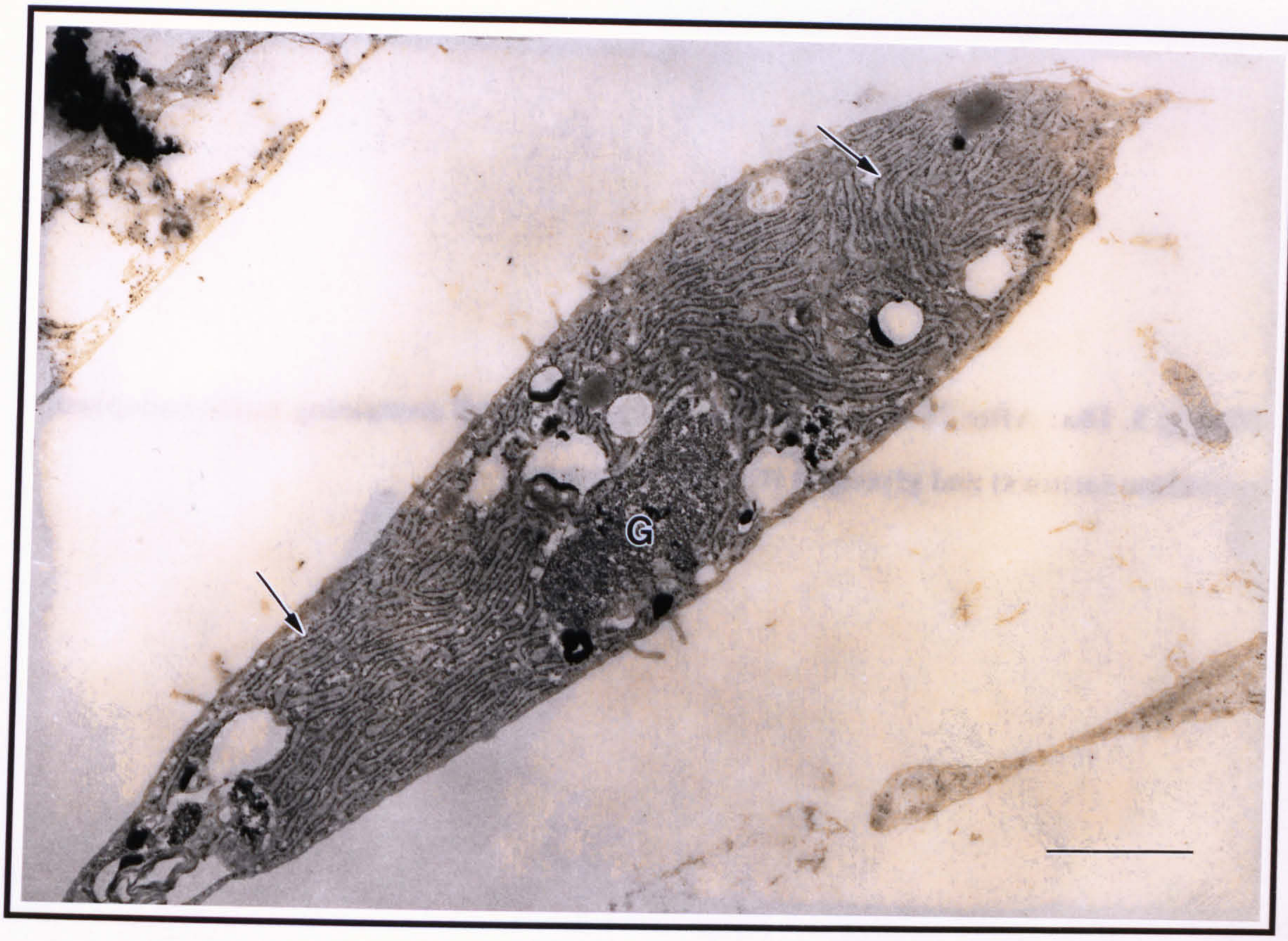


Figure 3.18a

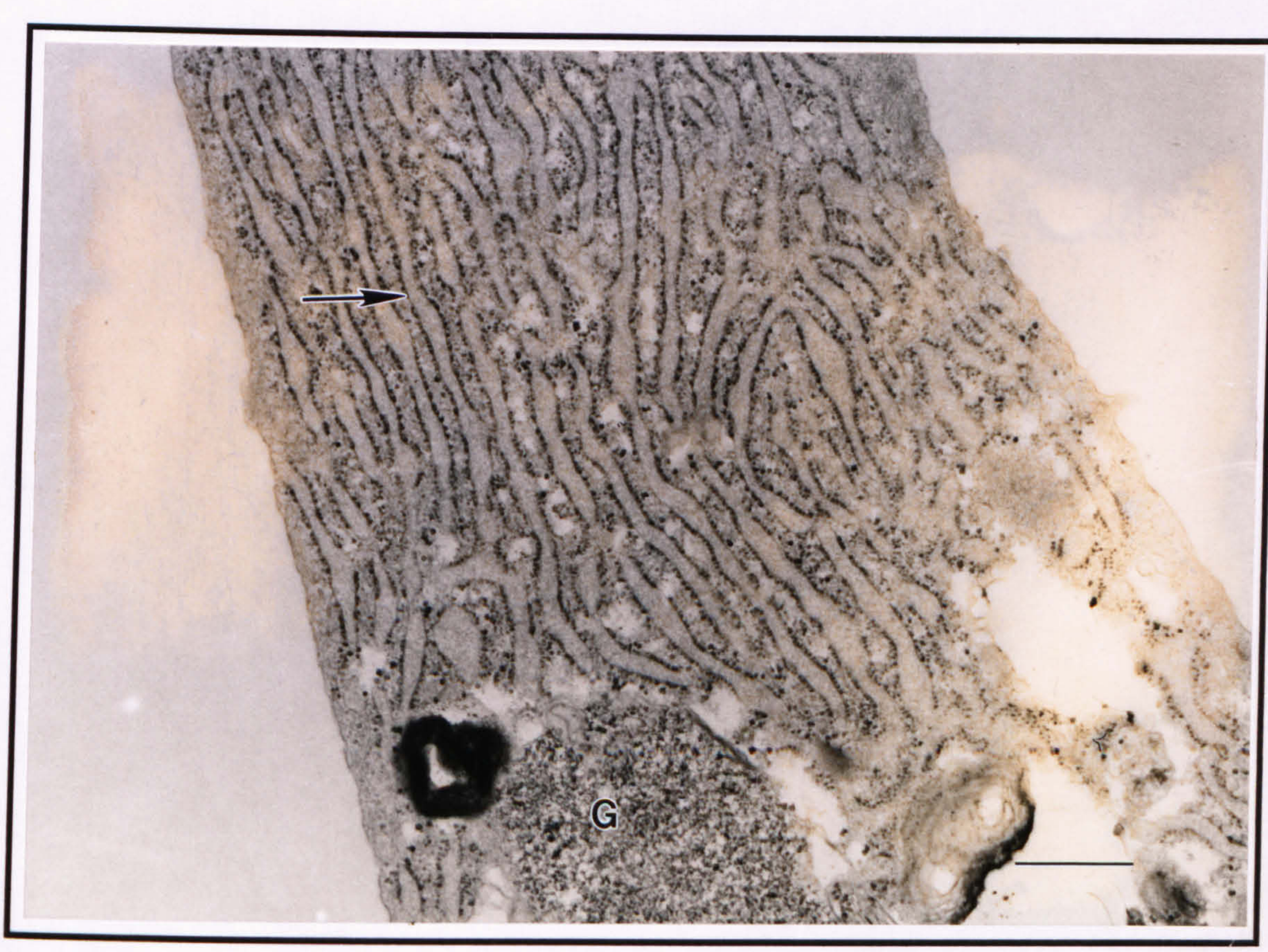


Figure 3.18b

Figure 3. 19a: After 28 days in bioreactor culture there is evidence of collagen (C) associated with the cells. These cells are observed to contain lipid droplets (arrows) in additions to other organelles as seen previously in figure 3.16. Bar = 3μm.

Figure 3. 19b: Collagen fibres (C) seen at higher magnification, showing evidence of early periodicity (arrows). Bar = 1μm.

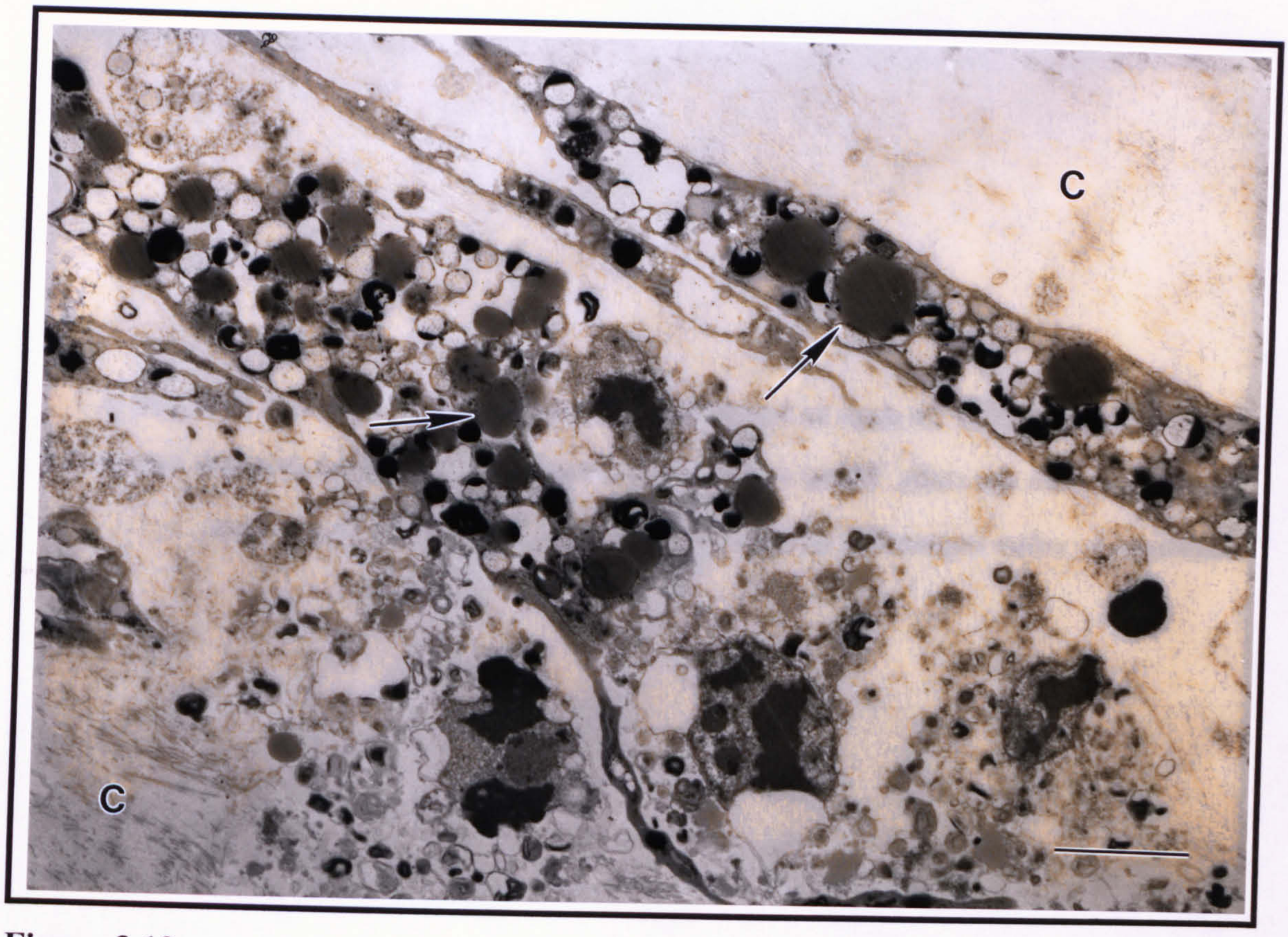


Figure 3.19a

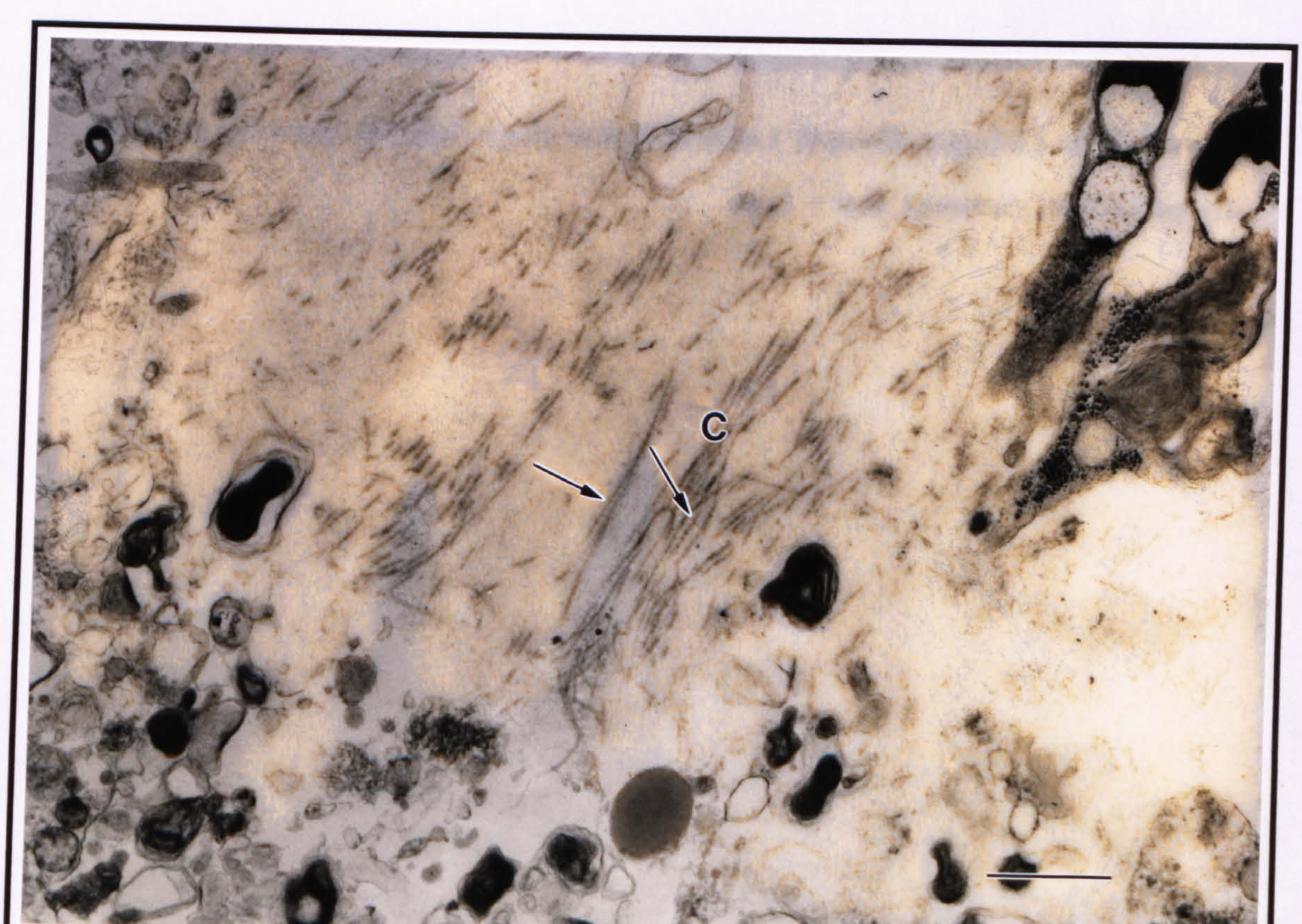


Figure 3.19b

Figure 3. 19c: Higher magnification of early collagen fibrils in the areas between cells. The periodicity of the collagen fibres (arrows), although faint, can still be seen. Bar = 250nm.

Figure 3.19c

Figure 3. 20a: Further illustrations of HA scaffolds cultured with MSCs in the bioreactor for 28 days. A number of cells are seen stacked together and extracellular collagen matrix (arrowheads) is noted between them. Several cells contain nuclei (N) and another contains glycogen (G) in close proximity to lipid droplets (L). Bar = 1μm.

Figure 3. 20b: Higher magnification of an area between two cells with nuclei (N), showing collagen (arrowheads) formation. Mitochondria (M) are seen in these cells. Bar = 0.5μm.

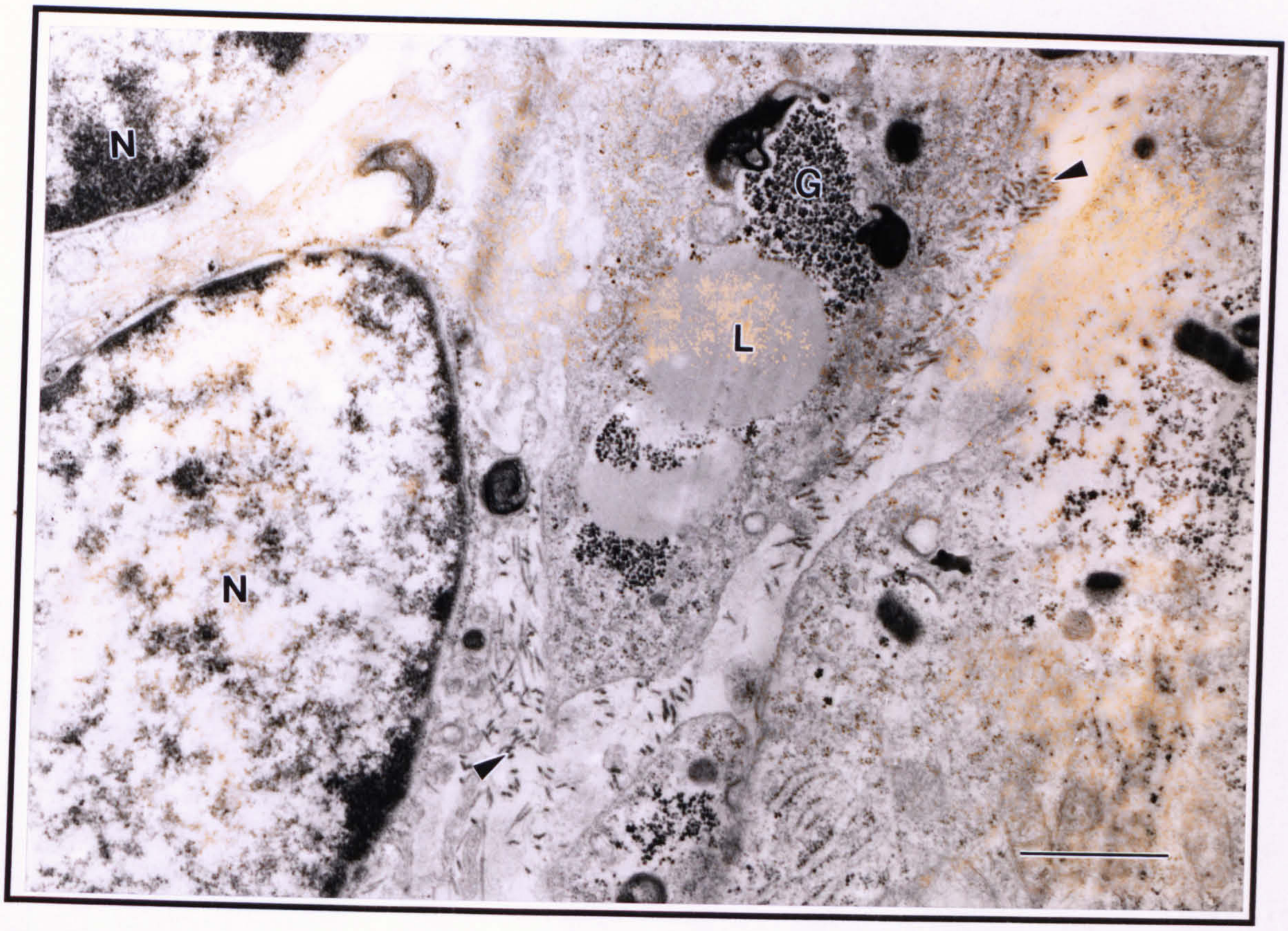


Figure 3.20a

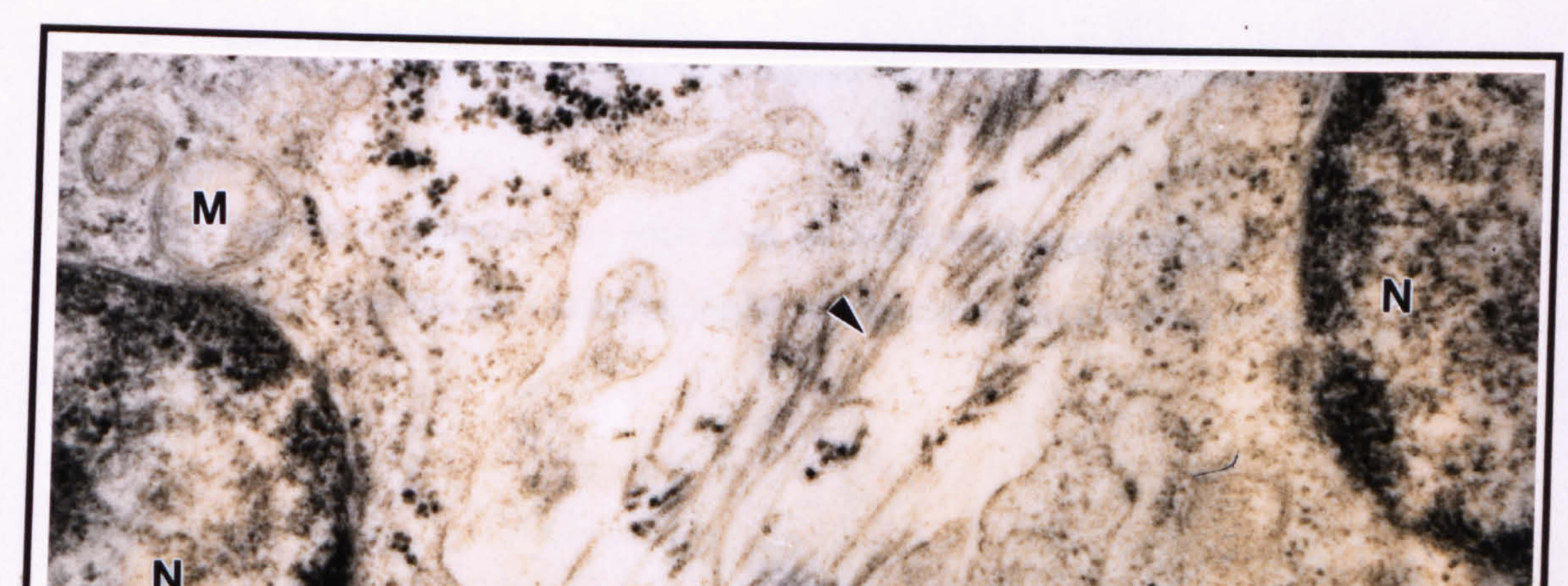


Figure 3.20b

Figure 3. 20c: Showing early collagen (C) between two cells again after 28 days on a separate HA sample in the bioreactor. Bar = 0.5μm.

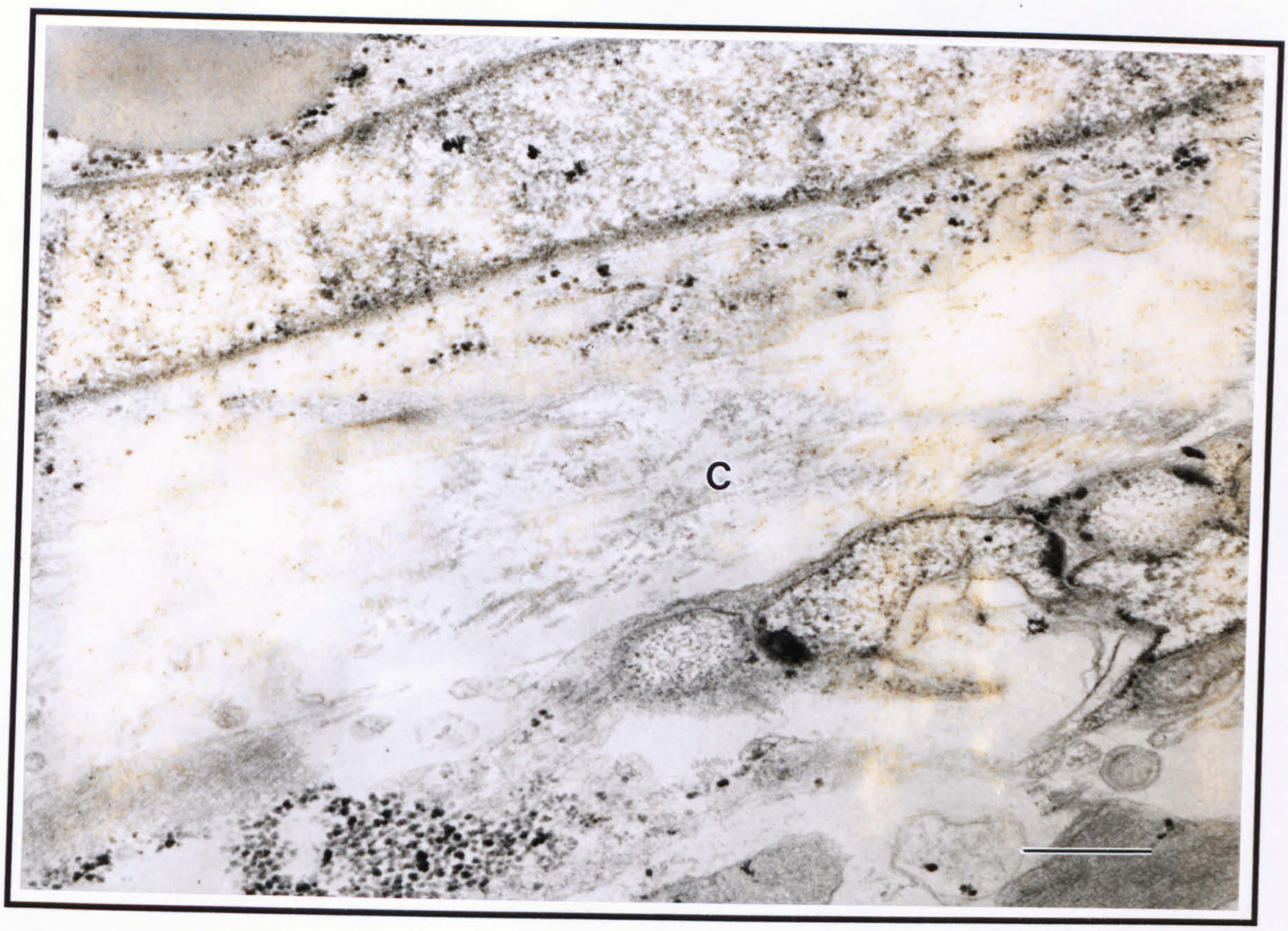


Figure 3.20c

Figure 3. 21a: Shows high magnification of a cell producing collagen (C) in close contact with a mineral deposit (Mn), after 28 days in the bioreactor. Arrows show the cell membrane and the cell contains an organelle suggestive of a mitochondrion (M). Bar = 200nm.

Figure 3. 21b: Shows lower magnification of cells in close contact (arrows) with a similar deposit as seen in figure 3.21a. The pointer indicates the area that was probed with the EDAX. Bar = 0.5μm.

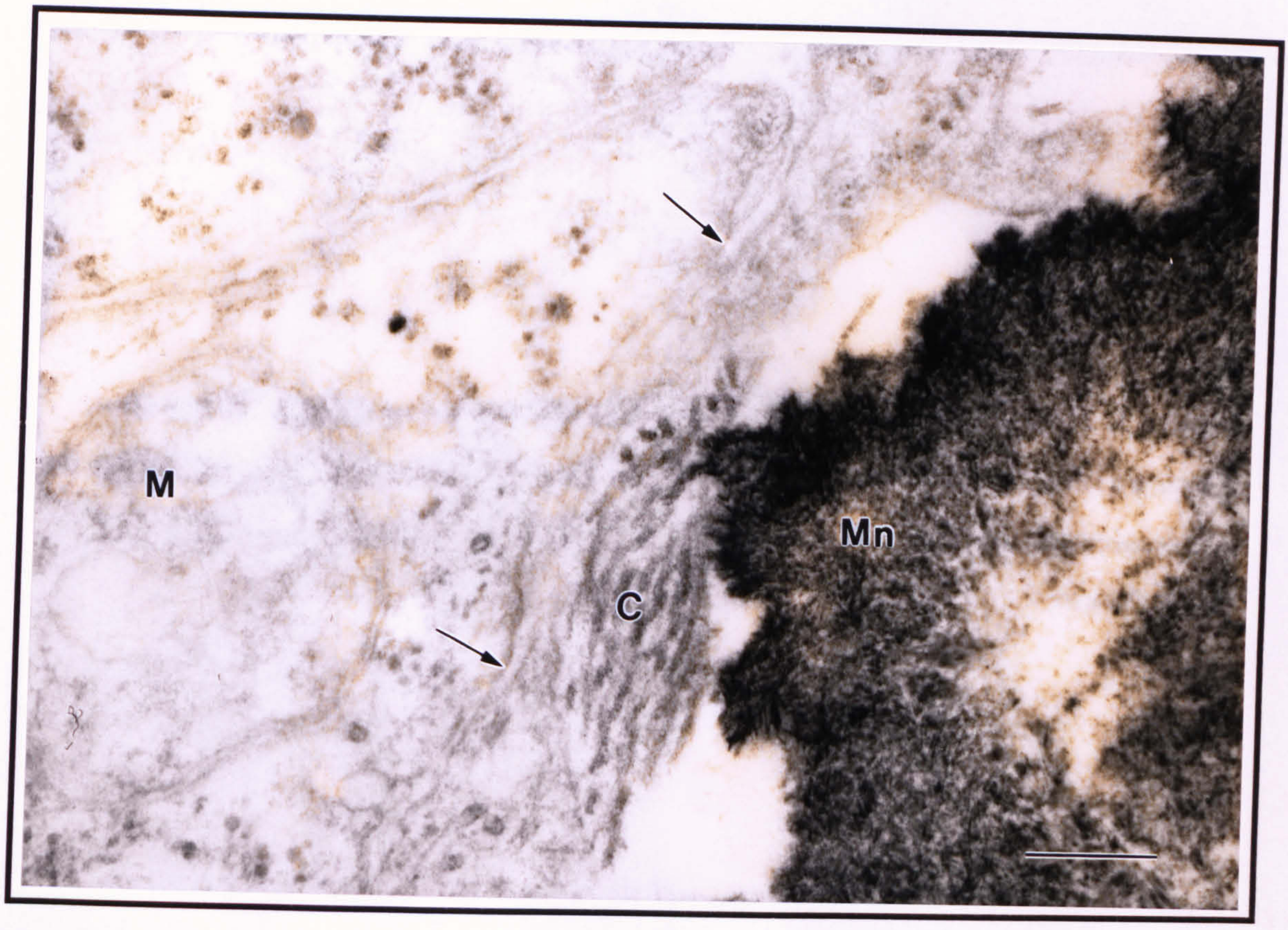


Figure 3.21a

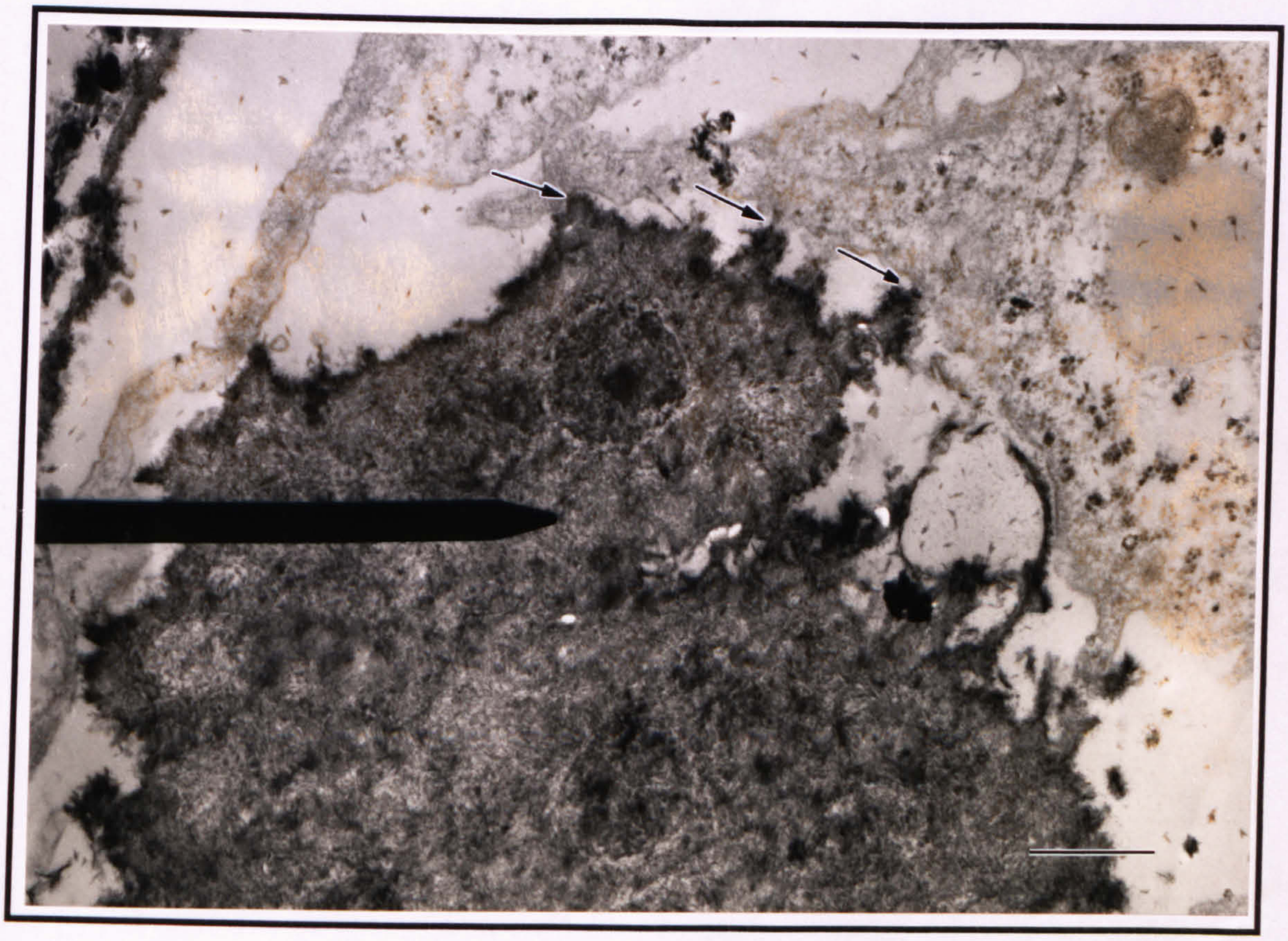


Figure 3.21b

Figure 3. 21c: Shows the spetra as analysed by the EDAX probe of the area indicated in figure 3.21b. Note the high content of calcium and phosphate.

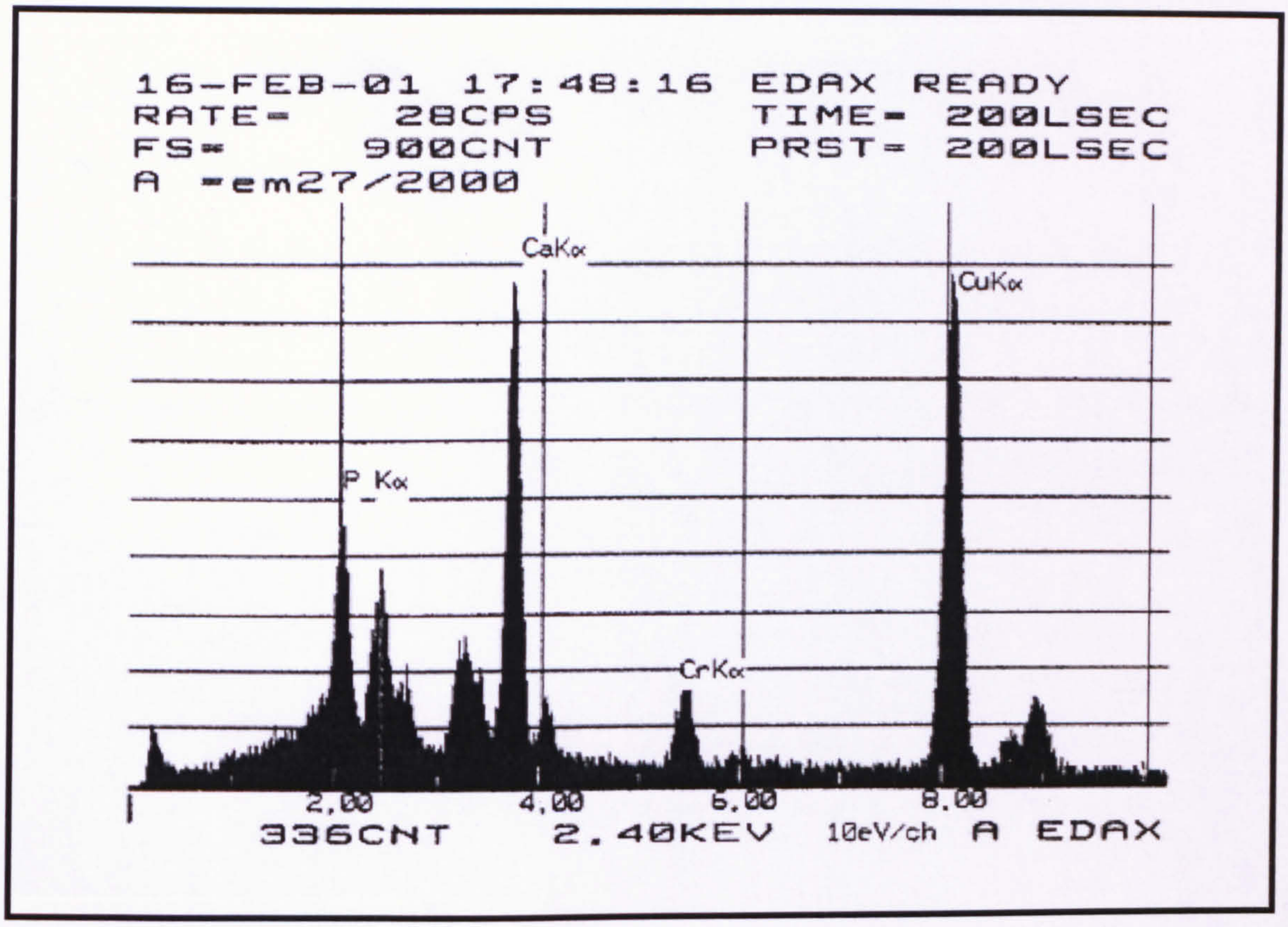

16-FEB-01 17:48:16 EDAX READY
RATE= 28CPS
TIME= 200LSEC
FS= 900CNT
PRST= 200LSEC
A =em27/2000
CaKα
CuKα
P Kα
CrKα
2.00
4.00
6.00
8.00
336CNT
2.40KEV
10eV/ch
A EDAX

Figure 3.21c

Figure 3. 22: In static culture, after 28 days cells were observed to contain a nucleus (N) and rough endoplasmic reticulum (arrow). Bar = 1μm.

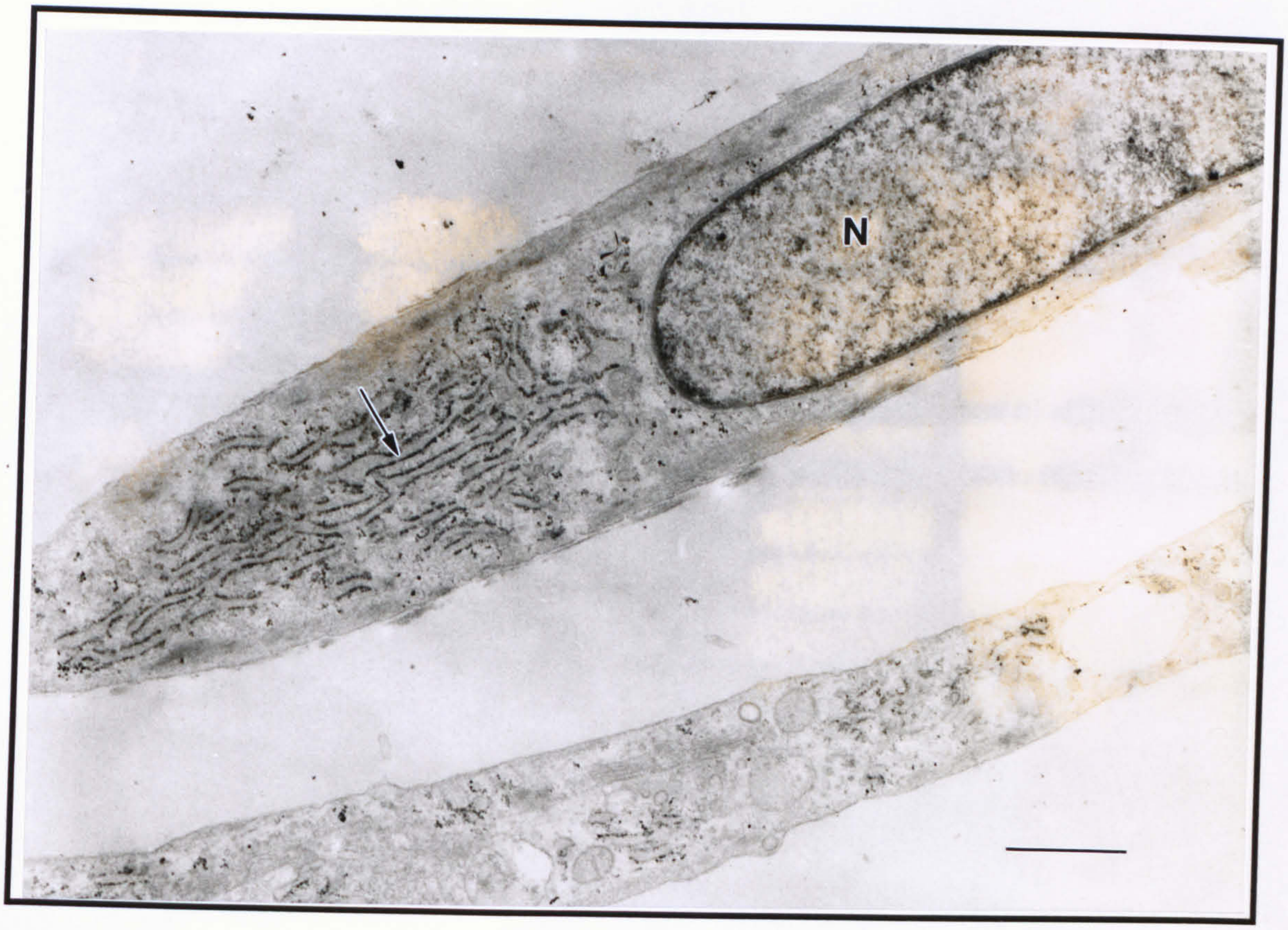


Figure 3.22

Figure 3. 23a: After 28 days in static culture on HA, there was the suggestion of collagen formation between cells (arrows). Bar = 1μm.

Figure 3. 23b: At higher magnification the cells were observed to contain rough endoplasmic reticulum (arrowheads) in addition to nuclei (N), but these cells possessed fewer cell processes than those cultured in the bioreactor (see figure 14). Bar = 0.4μm

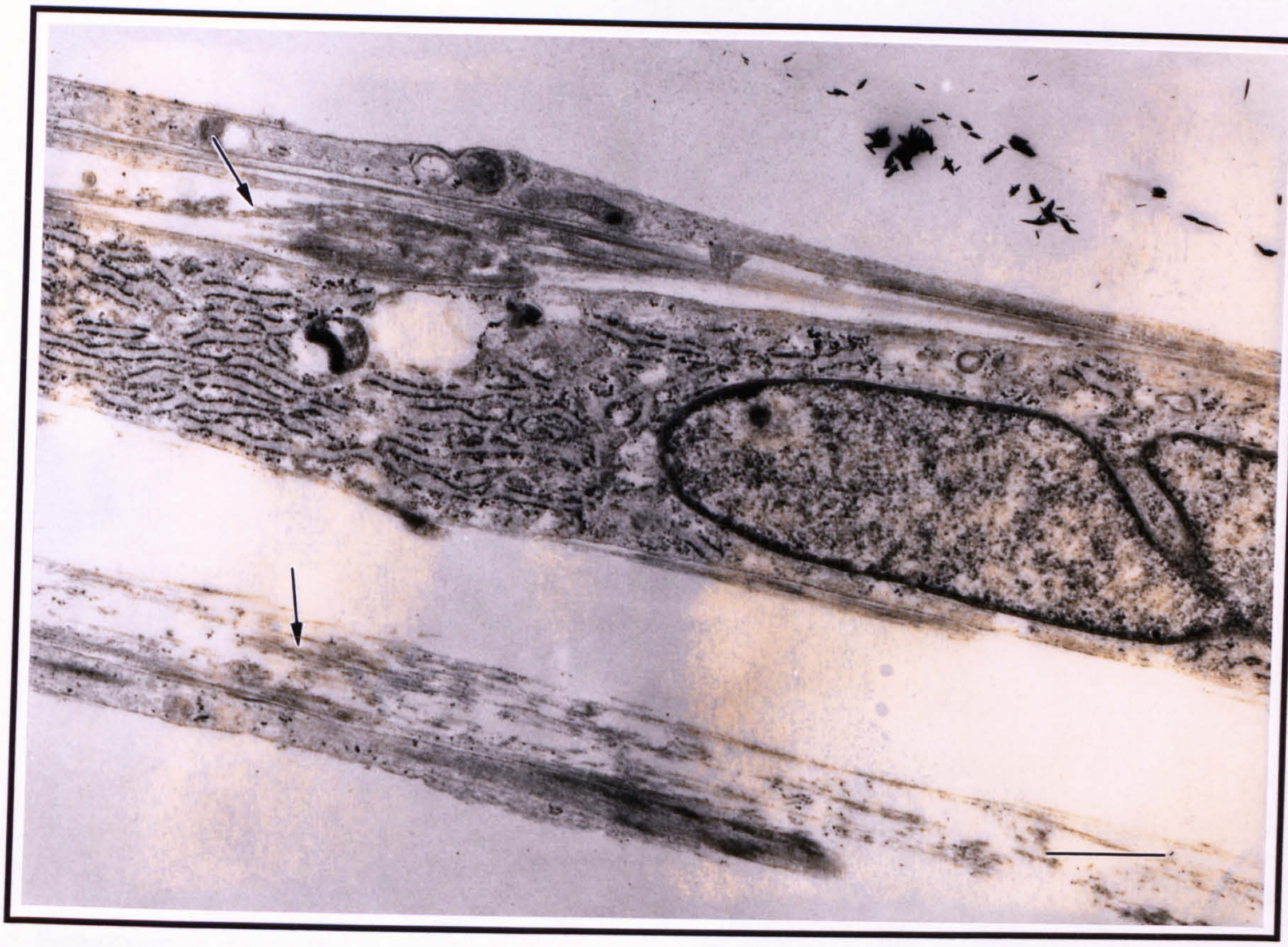


Figure 3.23a

Figure 3.23b

4.3.3.3 Messenger RNA RT-PCR results

The housekeeping gene GAPDH was expressed by all MSCs cultured with HA irrespective of culture condition (see figure 3.24).

The mRNA expression of Cbfa-1 was measured for MSCs cultured in the bioreactor compared with the static control, as a marker of differentiation down the osteoblastic lineage. MSCs on HA in both conditions expressed Cbfa-1 at 1, 2 & 4 days in culture (see figure 3.24a). In the bioreactor system the relative intensity of expression was greatest after the first day and reduced by day 4. However, in static culture the expression of Cbfa-1 was less intense initially but remained constant over the 4 days.

ALP expression was maximal after 1 day in bioreactor culture and decreased by day 4. However, it increased over the culture period in static culture, being maximal after 4 days in these conditions.

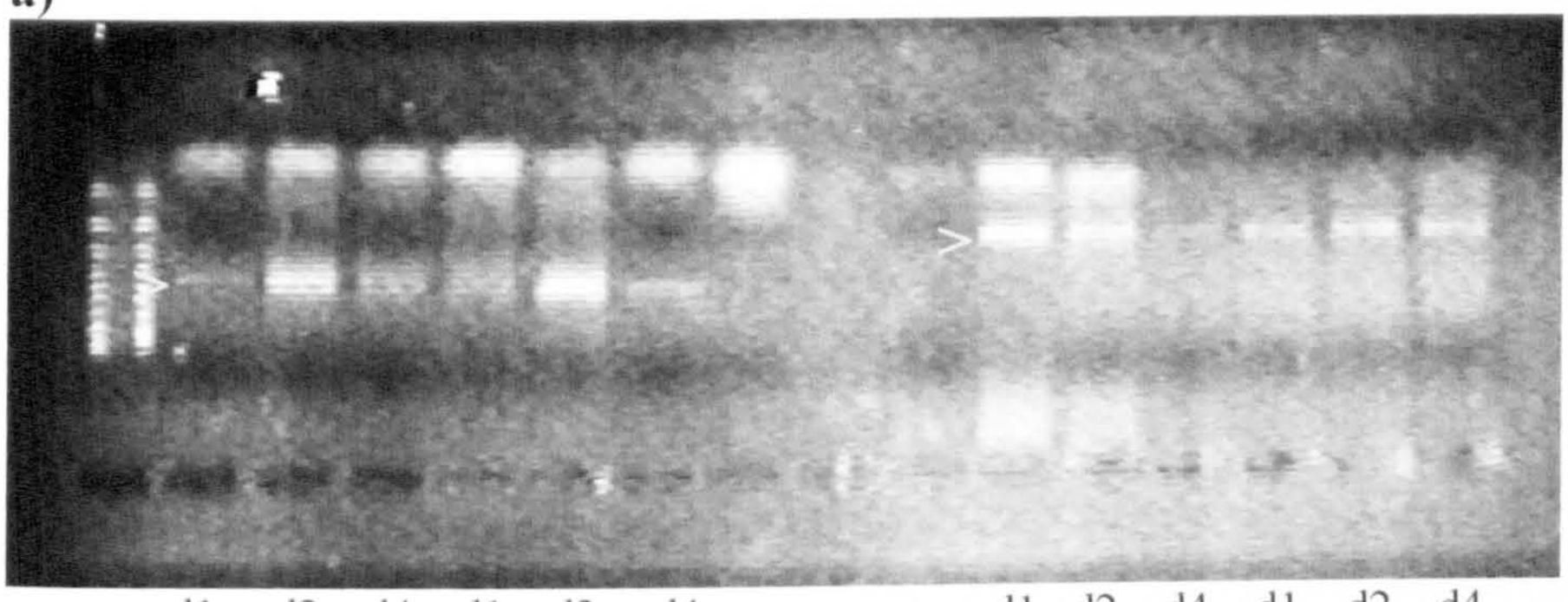
Osteopontin intensity varied slightly as it increased after day 2 in the bioreactor, but was greatest after day 4 in static culture (see figure 3.22b).

The intensity of osteocalcin expression was lower compared with the other mRNA tested. It was expressed after day 2 in both static and bioreactor culture and there was no apparent difference in expression between conditions.

MSCs cultured on HA expressed bone sialoprotein (BSP) after 1 day in either culture conditions (see figure 3.24c).

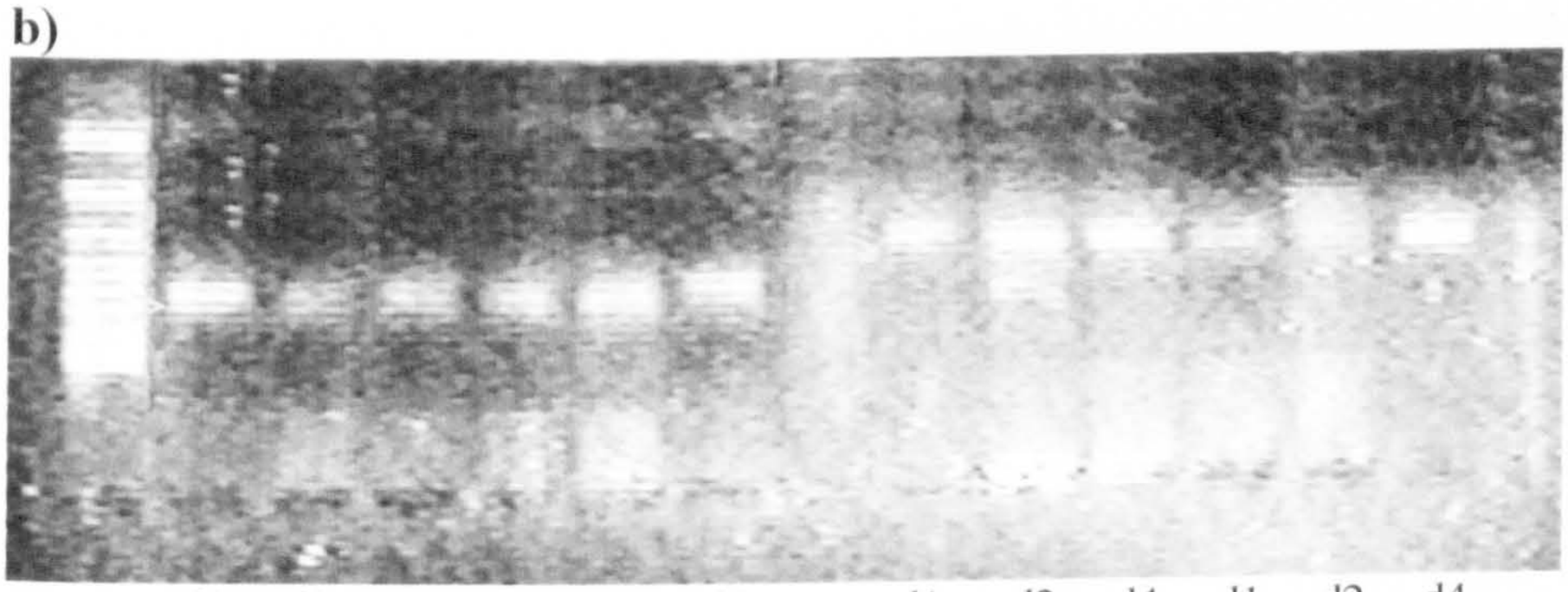
Figure 3. 24: Gene expression of MSCs cells cultured over 4 days on HA in the bioreactor compared with the static culture control, a) GAPDH & Cbfa-1, b) ALP & osteopontin & c) osteocalcin & BSP, yellow arrows indicate DNA bands.

a)



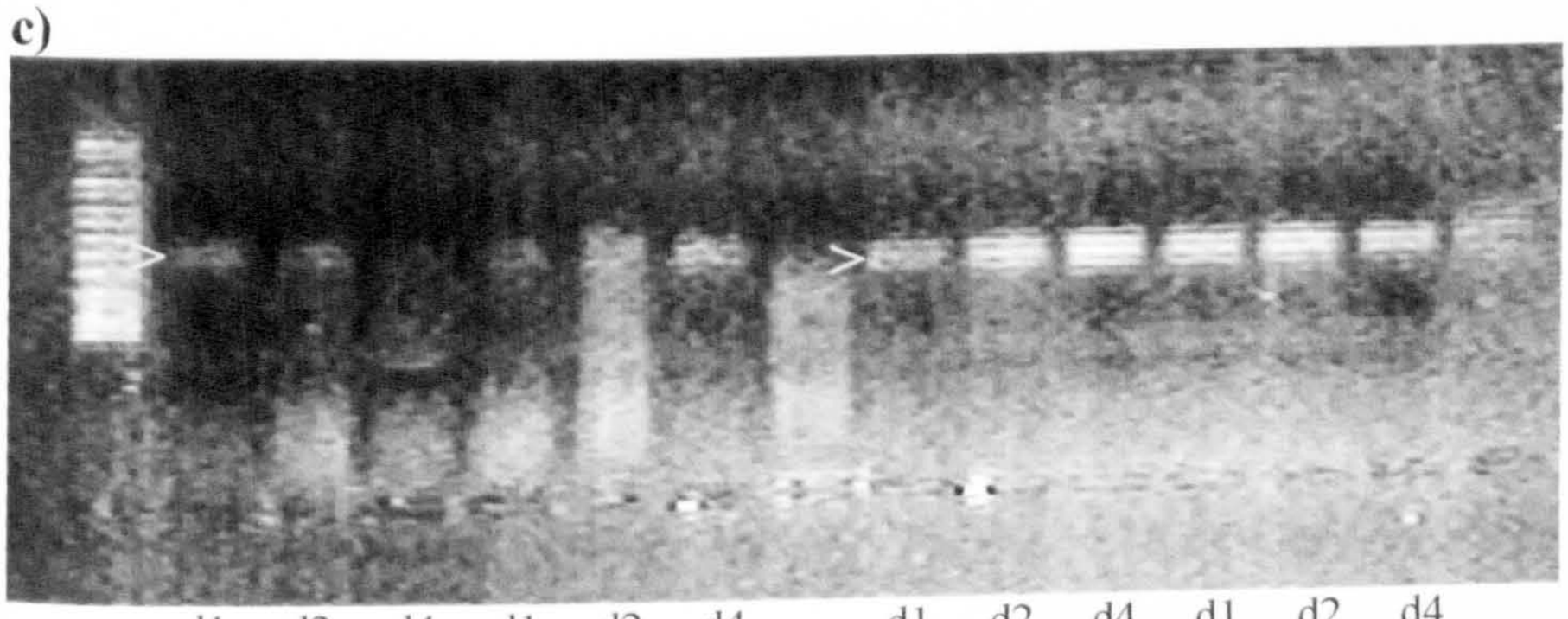
Ladder	d1 Bioreactor GAPDH	d2	d4	d1 Static	d2	d4	Blank	Blank	d1 Bioreactor CBFA-1	d2	d4	d1 Static	d2	d4

b)



Ladder	d1 Bioreactor ALP	d2	d4	d1 Static	d2	d4	Blank	d1 Bioreactor Osteopontin	d2	d4	d1 Static	d2	d4	Blank

c)



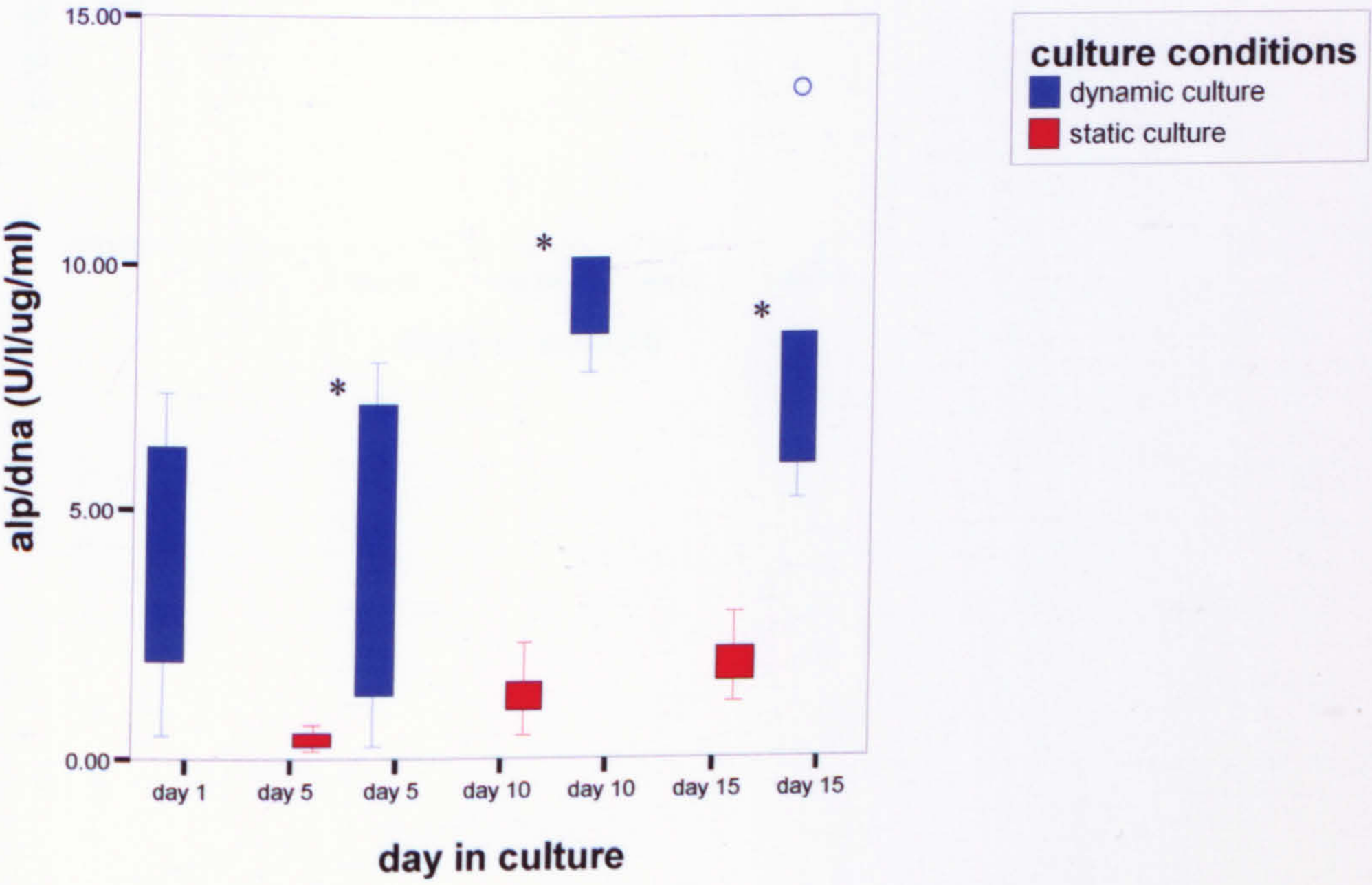
Ladder	d1 Bioreactor Osteocalcin	d2	d4	d1 Static	d2	d4	Blank	d1 Bioreactor BSP	d2	d4	d1 Static	d2	d4	Blank

4.3.3.4 Comparisons of ALP protein production

The ALP synthesised by MSCs on HA scaffolds cultured in the two conditions over a 15-day culture period was compared, as an indicator of osteoblastic differentiation. The ALP/DNA results for the two conditions were compared for each time point. ALP levels were too low to be detected by the assay after 1 day in static culture, although significant amount were measured in the cells cultured for 1 day in the bioreactor.

Subsequent to this, MSCs cultured on HA in the bioreactor system were found to produce significantly more ALP/DNA at 5, 10 & 15 days in culture ($P<0.005$), (see figure 3.25). The production of ALP by the MSCs cultured in the bioreactor peaked after 10 days in culture, however in static culture the ALP continued to increase throughout the culture period.

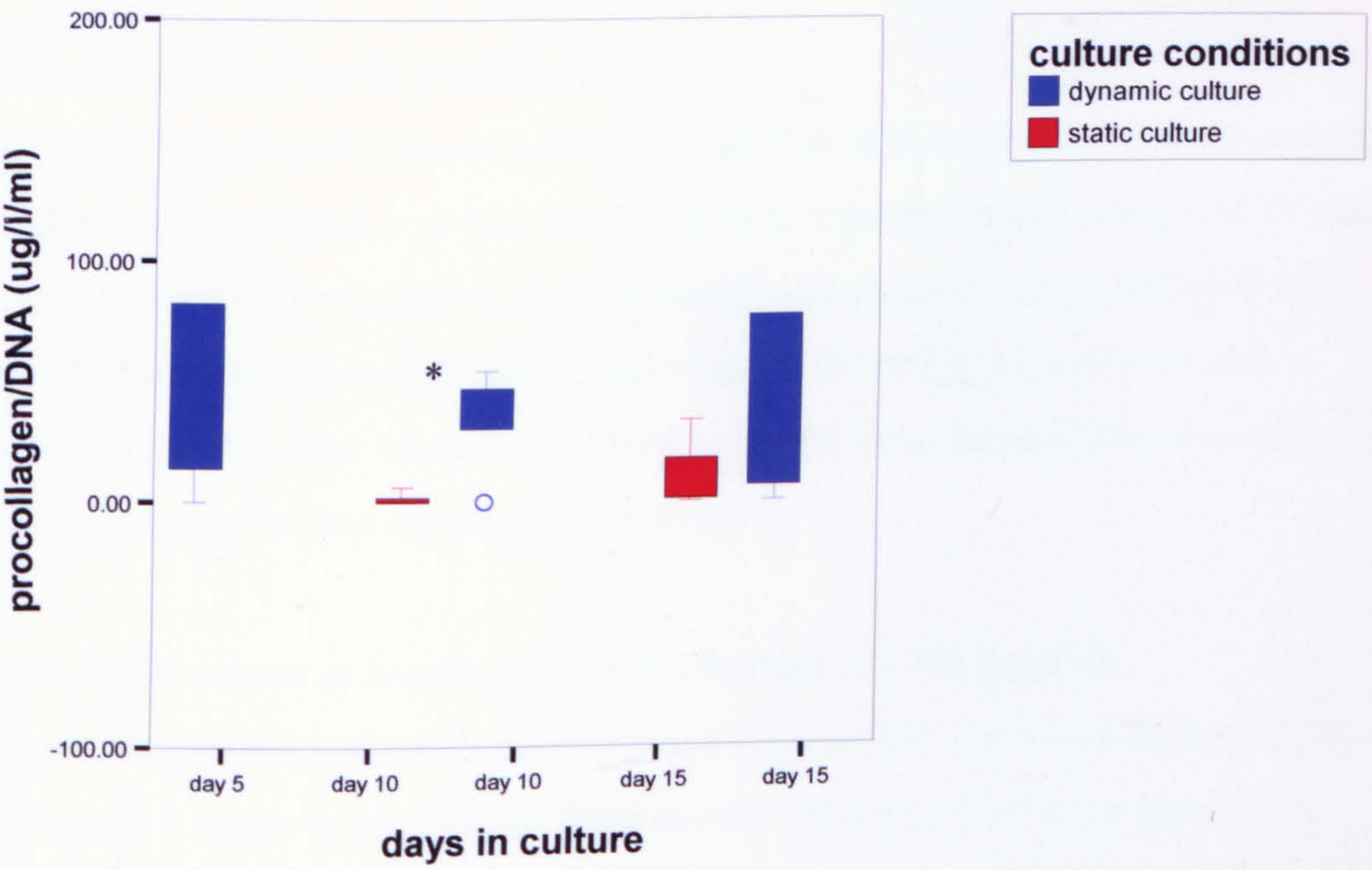
Figure 3. 25: The production of ALP/DNA over the 15-day culture comparing static and bioreactor (dynamic) culture conditions, * P-value <0.005.



4.3.3.5 Comparisons of Type I Procollagen protein (PICP) levels

The amount of PICP/DNA cleaved from the peptide, by MSCs cultured on porous HA in the bioreactor after 5, 10 & 15 days, was compared with the static culture control. A significantly larger amount of PICP was produced by the MSCs in the bioreactor after 10 days in culture ($P<0.05$) and no PICP was detected in the cells after 5 days in static conditions (see figure 3.26).

Figure 3. 26: Box plot of the PICP production by MSCs cultured on porous HA within the bioreactor compared to static culture over 15 days, * $P<0.05$.



4.4 DISCUSSION

The aim of Chapter 4 was to design a novel bioreactor to aid the culture of MSCs on HA scaffolds and to encourage the differentiation of MSCs with the production of osteoblastic extracellular matrix. The design of the bioreactor was based on the theory that if nutrients flowed over cells through a porous scaffold, it may increase the differentiation of MSCs into osteoblasts at a greater rate than observed in static culture conditions as demonstrated in Chapter 3. Furthermore, this flow of medium was also suggested to increase the penetration and survival of cells through the scaffold, thereby tissue-engineering a 3-d construct.

4.4.1 Penetration of cells through the HA scaffold

4.4.1.1 Cell numbers in cross section

The first of this chapter's hypotheses proposed that MSCs cultured in my bioreactor would penetrate through a porous HA scaffold to a greater extent compared to static culture. The result of measuring the number of cells through cross-sectioned HA scaffolds confirmed this after both 7 and 14 days, ($P<0.05$). This leads to the conclusion that the flow of medium through the HA does increase the penetration of the cells and allows their survival for 14 days.

4.4.1.2 Cell counts at increasing depths through the HA scaffold

To assess further the cell penetration through the scaffold, the cross-sectioned HA was divided into 5 layers at intervals of 2mm depths. When each of these layers was assessed, it was observed that after 7 days in the bioreactor, cells had penetrated all the way through the HA, maintaining a large cell number to 8mm depth. Furthermore, penetration of MSCs to 4, 6 and 8mm through the porous HA cultured in the bioreactor was greater than in static culture, after 7 days ($P<0.05$). As cells continued to be observed through the scaffolds after 14 days in the bioreactor, this culture system maintained cells throughout the scaffold confirming that cells penetrated the HA and received enough nutrients and oxygen to remain viable.

In static culture after 7 days, however, very few cells penetrated beyond the first 2mm into the HA scaffold. Explanations for these findings are that, in static culture,

penetration into the HA was slow and limited. Moreover, there was evidence of cell debris presence at 6mm depth when observed under SEM, which suggests that cells present in the centre of the scaffold were unable to survive. This was likely to be due to an insufficient supply of nutrients, which relies on diffusion in static culture conditions.

After 14 days in static culture, cells had penetrated slightly further through the scaffold, to a depth of 4mm but, like the findings at day 7, more MSCs were seen through the remaining layers (6-8mm) when cultured in the bioreactor ($P<0.05$).

The number of cells present within the top layer of the HA scaffolds did not vary significantly between the two conditions after 7 days. This is because the cells seeded onto the HA in static culture would have initially adhered to the surface of the scaffolds and proliferated to cover the local area of the HA. Furthermore, similar cell survival would be expected as supply of nutrients should be adequate in each condition. Additionally it should be noted that, as the number of cells counted within the top layer varied considerably and the graph had large error bars caution is required in interpretating this result.

After 14 days there was no significant difference in the number of cells in the second layer (4mm) between the two culture conditions, but significantly more cells in the top layer (2mm) of the bioreactor culture. This suggests that some of the cells from the top of the static culture scaffold have moved down through the scaffold into the second layer. This may be the result of cell migration, although, a further explanation could be that cells proliferated within the top layer and spread to the second by 14 days in static culture. However, as large error bars were also present on the graph for the second layer, this result needs to be cautiously interpreted.

Furthermore, as cell debris was observed at the deeper layers of static cultured HA after 7 days, but less debris was evident by day 14 following static culture. This suggests that cells penetrated the static cultured scaffold early, possibly at the time of seeding, but in static culture these cells were unable to survive. As there was no further evidence of cell debris by day 14, this indicates that once in static culture cells did not move through the scaffolds.

The increased cell penetration in the bioreactor was likely to be the result of cell division and movement, due to the flow of medium stimulating the migration of cells through the porous HA scaffold. Once within the scaffold the flow of fresh nutrients maintained these cells, allowing them to survive and proliferate covering the surface. Further investigation is needed to study the methods of cell movement through the scaffolds in the two culture conditions.

Therefore, the bioreactor system increased the density of cells through the HA scaffold forming a 3-d cellular construct. Thus, this method would increase the potential for the use of this MSC-HA scaffold in clinical bone grafting procedures.

4.4.3 Assessment osteoblastic differentiation of MSCs

4.4.3.1 Observations under SEM

The second hypothesis tested in this chapter was that the bioreactor culture system stimulated the differentiation of the MSCs into osteoblasts. This was initially assessed by comparing the morphology of cells in the two culture environments under SEM. By day 14, the morphology of the cells cultured on HA in the bioreactor was observed to change from spindle-shaped cells, characteristic of fibroblasts, to plump, cuboidal cells, typical of osteoblastic morphology (Holtrop 1990).

MSCs grown in static culture were also shown to have altered morphology by day 14, as seen previously in Chapter 3. In both culture systems cells formed multiple attachments to the HA via cell processes. The change in shape is therefore suggested to be due to the HA, as shown in Chapter 3, although the rate of change seemed to be more rapid when cells were cultured in the bioreactor.

4.4.3.2 Observations under TEM

Prior to observing the cells under TEM, the scaffolds were viewed under light microscopy. The results of this illustrated a dramatic difference in the numbers of cells on the HA depending on culture conditions. This also supported the results of the cell penetration study (see above). When the constructs had been grown in the bioreactor cells were seen to fill the pores of the scaffold, however after a similar culture period

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in static culture fewer cells were seen. As the cells formed a mesh filling the pores of the HA following culture in the bioreactor, it is probable that an extracellular matrix would form between the cells. In the case of osteoblasts this would be bone and therefore, my bioreactor would be useful for the growth of tissue.

TEM was then used to examine the cell ultrastructure and the extracellular matrix produced by cells cultured on the HA in both static and bioreactor culture. Although cells were seen in close contact to the HA, it was difficult to examine their attachment as the electrons from the microscope caused the HA crystals to break up, resulting in the scaffold and cells moving under the beam. As under SEM, the cells were observed to possess many cell processes, suggesting that MSCs attach to HA.

Cells cultured on HA in the bioreactor for 14 days contained many organelles including mitochondria, which produce cellular ATP and are associated with fatty acid and protein synthesis. Large amounts of endoplasmic reticulum (ER) were also observed within these cells and at higher magnification this was shown to be rough ER, due to the ribosomes seen studding it. The main function of rough ER is to produce secretory proteins, which would include extracellular proteins such as osteopontin and isoenzymes such as ALP. This suggests that the cells cultured in the bioreactor for 14 days were consistent with active cells producing proteins.

After 28 days the cells were also noted to contain large amounts of intracellular glycogen, which is uses as intracellular energy stores and are commonly seen adjacent to rough ER in liver cells (Ham 1974). These observations indicate that the cells were metabolically active, which is typical of functioning osteoblasts (Holtrop 1990).

Intracellular lipid droplets were observed in some of the cells cultured for 28 days in the bioreactor. Small lipid inclusions are found in most animal cells (Ham 1974). Lipid can be an important storage method for cells in culture and the larger amounts seen in cells grown in the bioreactor indicates that the cells were well supplied with nutrients.

Furthermore, after 28 days in the bioreactor, there was evidence of collagen production throughout each of the scaffolds studied. Notably this was seen between cells and it was organised, in some cases with periodicity and early striations observed on the

fibrils. As collagen matrix matures it becomes organised with striations of the fibres, the thickness of the collagen then increases forming the basis of osteoid that is mineralised to produce bone. There was evidence that the collagen produced by cells in my bioreactor was starting to mature. However, experiments over a longer culture period would be required to further examine extracellular matrix maturation.

There was also evidence of mineral formation that, on EDAX probing, had average calcium to phosphate ratio of 1.8. As the ratio of HA, the inorganic component of bone, is said to be 1.67 this may represent mineralisation, thereby clarifying the presence of functioning osteoblasts. However, although the mineral was observed adjacent to collagen matrix, the matrix was not as dense and mature as would be expected adjacent to mineralisation. Furthermore the mineral was observed to have crystal shaped structures around its edge, which is not characteristic of mineralised extracellular matrix. As the background EDAX absorbance ratio was also high, this suggests that the EDAX ratio of this mineral may actually be slightly lower, signifying HA scaffold. Nevertheless, this mineral was not typical of the HA scaffold when observed under TEM, although cell processes were seen attaching to it as they tend to do to HA.

In conclusion, further experiments are necessary to test whether this mineral was produced by the cells or whether was derived from the HA scaffold. In addition to this, as 28 days is early for mineralised osteoblastic extracellular matrix, supplementary culturing in the bioreactor over a longer time period should be studied.

In static conditions, after 14 days in culture on HA, there were not enough cells present to be visualised under TEM, as observed when scaffolds were placed under light microscopy. In comparison to the bioreactor culture this is explained by a lower penetration and proliferation rate found in cells in static conditions.

After 28 days in static culture metabolically active cells were observed, as seen by the presence of organelles and rough ER (Bab et al. 1988a) and there was evidence of collagen production on one of the scaffolds. However, the extracellular matrix was less frequent than that observed when the cells were cultured in the bioreactor. This would

be expected from the lower density of cells observed under light microscopy prior to TEM.

In summary, these results highlight that culture in the bioreactor stimulated MSCs to become active, producing early extracellular matrix of osteoblasts to a greater extent than in static conditions.

4.4.3.3 Messenger RNA

The gene expression of ALP, osteopontin, BSP and osteocalcin, all markers of osteoblasts, and Cbfa-1, a marker of osteoblast precursor cells, by MSCs cultured on HA in the two conditions was assessed by RT-PCR. The success of the technique used was confirmed, as the housekeeping gene GAPDH was expressed by every sample in both conditions.

Cbfa-1 is a transcription factor that is necessary for the expression of osteocalcin and can therefore be used as a marker of MSCs differentiating into osteoblast progenitor cells (Ducy et al. 1997; Ducy 2000). The expression of Cbfa-1 increased until day 4 in static culture, whereas it reduced after 1 day for cells grown in the bioreactor. This result indicates that in both cases the MSCs differentiated down the osteoblastic cell line and that this occurred at an earlier stage in the bioreactor culture relative to the static control.

ALP and osteopontin were also expressed earlier by MSCs cultured on HA in the bioreactor relative to static culture. This also suggests that MSCs cultured in the bioreactor differentiated into osteoblasts earlier than in static conditions. Osteocalcin was expressed in both culture conditions increasing until day 4. The weak expression is due to it being a late marker of mature osteoblasts. Similarly there was no notable difference in BSP between the two conditions. Osteocalcin, as discussed in Chapter 2 Results, is distinctively associated with mineralised bone tissue (Aubin 1998) and BSP is an attachment protein for collagen that is found exclusively in bone (Oldberg et al. 1988b). In summary, this is evidence of the presence of the osteoblastic phenotype within the cultures, with expression of certain osteoblastic markers occurring earlier in bioreactor culture.

4.4.3.4 ALP protein production

Protein production is a more accurate marker of cell phenotype than mRNA as, although transcription is necessary for protein synthesis, it does not ensure that the cell will produce the protein. Therefore, ALP protein levels were measured in addition to mRNA synthesis.

Production of ALP was used, as it is a marker of the osteoblastic phenotype (see Chapter 1) and as it was shown to increase as MSCs differentiated into osteoblasts in Chapter 2. After 1 day in static culture, the levels of ALP were too low to measure, however significant amounts were measured for cells cultured in the bioreactor. Following this, the amount of ALP synthesised by the cells was significantly greater after 5, 10 and 15 days, for those cultured in the bioreactor compared to the static culture control ($P<0.005$). As the levels were standardised for DNA content, this is likely to be the result of greater ALP production at a cellular level in bioreactor conditions. Overall, these results show that use of the bioreactor increased the rate of differentiation and osteoblastic activity of the MSCs, which furthermore supports the ALP mRNA results.

As described in the Introduction to this chapter, my bioreactor was designed to simulate the conditions of osteoblasts *in vivo* with the aim that this would stimulate cell differentiation and production of bone. The greater increase in osteoblastic function at a more rapid rate by the MSCs cultured on HA compared to static culture, may be the direct result of the bioreactor system stimulating osteoblastic differentiation.

However, this increased rate may also be due to the increased mass transfer of nutrients as a result of the use of a bioreactor, as seen when osteosarcoma cells were cultured in suspension in a RWV (Granet et al. 1998). Hence in my bioreactor, the quality of nutrient supply may have promoted cellular activity allowing cells to proliferate, whereby increasing cell differentiation as stimulated by the HA scaffold, as shown in Chapter 3. Conversely, in static conditions the supply of nutrients to cells within the scaffold by diffusion may have limited cell growth and thus differentiation.

4.4.3.5 Type I Procollagen (PICP) protein production

Type I procollagen is produced by osteoblasts, following which it is converted into type I collagen by the removal of the carboxyterminal propeptite (PICP), which allows organisation into fibrils forming the basis of osteoid. Thereby, the concentration of PICP found is proportional to the levels of recently synthesised type I collagen. Detection of PICP has been described as the technique of choice for quantification of type I collagen synthesis by active osteoblasts *in vitro* (Nacher et al. 1999). Therefore in this study, PICP levels were measured as a specific protein marker of osteoblastic extracellular matrix production, the result of MSCs differentiating into osteoblasts.

It was found that PICP was produced between 5 and 15 days, when MSCs were cultured on HA in the bioreactor. In contrast, PICP was not detected until after 10 days in static culture. At this point, levels of PICP produced in the bioreactor were significantly higher than in static conditions ($P<0.05$), indicating that the bioreactor MSCs had been stimulated to differentiate into osteoblasts and synthesise extracellular matrix earlier. However, after 15 days, there was no significant difference between the amounts of PICP produced by cells in the bioreactor compared with the control, suggesting MSCs in static culture, had differentiated and also started producing extracellular matrix like those in the bioreactor. These results confirm the observations under TEM, whereby cells cultured in the bioreactor produced larger amounts of collagen matrix, earlier in culture. Although it was also demonstrated, by these results and under TEM, that MSCs cultured on HA in static culture would produce collagen, suggesting differentiation into osteoblasts as shown in Chapter 3. This differentiation occurred at a slower rate than when cultured in the bioreactor.

4.4.4 Design of this bioreactor

Suggested benefits of the use of my bioreactor in tissue engineering of bone include:

1. A perfusion system which increased cell penetration through a scaffold and improved 3-d growth,
2. Flow through the scaffold increased the efficiency of nutrient and waste exchange, allowing increased survival of penetrated cells,
3. Peristaltic flow of medium simulating the physiological environment of bone, to stimulate the differentiation of MSCs into osteoblasts.

The aim of tissue engineering is to produce a tissue-like structure by growing cells on a scaffold that can be easily handled by a surgeon, for implantation as a graft to heal a tissue defect in a patient. Unlike previous bioreactors where the cell-scaffold constructs are moved, my bioreactor design enabled the stable housing of a 3-d scaffold, through which medium could perfuse. This produced suitable tissue-like osteoblastic cells, which could be differentiated from autologous or allogenic marrow cells.

The increased rate of differentiation of MSCs into osteoblastic cells was suggested to be due to the simulation of the physiological environment of osteoblasts in living bone. However, the increased osteoblastic activity, measured by protein and extracellular matrix production may also have been due to the increased mass transfer in this system. Although in either case, the system increased differentiation, which was its overall aim.

The flow through the bioreactor would not have been linear, as turbulence occurs when fluids flow through a tube and this would have been further increased by the presence of the scaffolds. The resulting shear forces generated within the bioreactor chamber would have had an effect on the cells, however the flow would still have increased the supply of nutrients to the cells. Furthermore, the penetration of cells through all surfaces of a porous HA increased, indicating that the turbulence did not adversely effect cell movement.

4.4.5 Bioreactor for commercial use

For this bioreactor to be used commercially, various additional points would need to be addressed. These include the assurance of the quality of the cell-scaffold constructs by monitoring the tissue grown for cellular structure and function to retain reproducibility. Indeed before commercial use for engineering graft material for patients, further animal studies would be needed to clarify the histological result of implanting such constructs into a bone defect. Further, for patient use the bioreactor components need to either have to be fully sterilised or disposable to prevent infection. The system should be automated as far as possible, but trained clinical staff would also be needed to operate the bioreactors.

CHAPTER 5

Storage of Fresh Mesenchymal Stem Cells Isolated from Bone Marrow

5.1 INTRODUCTION

5.1.1 Background to this chapter

As shown in Chapter 2, human bone marrow is a source of mesenchymal progenitor cells (MSCs) which, following isolation, can be differentiated into osteoblasts under appropriate conditions in culture. Therefore, bone marrow can be used as a source of autologous osteoblasts for potential implantation on compatible osteoconductive grafts to fill bone defects. One such potential use of MSCs on a graft material (HA) was investigated in Chapter 3, where it was found that bone marrow-isolated cells cultured on HA differentiated into osteoblasts. This provides a basis for tissue-engineered bone. Furthermore, in Chapter 4 this tissue-engineered bone was shown to be improved by culture in my bioreactor. However, as these techniques rely on a source of MSCs isolated from bone marrow, either autologous cells from patients or allogenic from a donor, this chapter will investigate a means of preserving such cells.

Cell viability can be maintained under culture conditions, but this is a labour intensive method of sustaining the cells' metabolic demands and, while in culture, the cells are also at risk of infection and biological variation due to genetic drift. So, to enable the clinical use of these tissue-engineering applications of MSCs, methods for storing the cells between harvest and delivery to the patient are necessary to avoid long-term tissue culture. As biological material has a limited shelf life and is open to contamination, simple refrigeration at 4°C is unsatisfactory, other methods of preserving and storing MSCs need to be investigated. One such method is cryopreservation which is an easy and effective means of storing biological material in liquid nitrogen as, below –120°C, cells have no metabolic needs and can therefore be stored for years.

5.1.2 Cryopreservation

Cryobiology is the study of life at low temperatures and cryopreservation is the maintenance of biological material in a state of suspended animation at cryogenic temperatures. The temperature of liquid nitrogen is –196°C and cells can be stored in this environment by the process of cryopreservation. This was first pioneered for the storage of spermatozoa in 1949 (Polge et al. 1949). Since then, cryostorage has been

found to be a suitable method of storage for a variety of living cells including cord blood, bone marrow, reproductive cells, and tissues such as skin, cornea and heart valves. Further research has made the current clinical practice of storing haematopoietic stem cells from autologous bone marrow possible, before engraftment following marrow ablation (Rowley SD 1992b).

In cooling the cells to sub-zero temperatures cells are at risk of lethal freezing or cold shock – cellular damage induced by rapid cooling without protection. However, cryopreservation methods limit this (Morris et al. 1983) and can allow up to 95% of cells to recover after resuscitation. Viability of the cells is measured by their ability to function to the same degree as prior to preservation, for example to produce a protein to the same extent.

Cryopreservation is a complex process that is still not fully understood, but follows the theory that cellular metabolism stops when all the water in the system is converted to ice (Rowley SD 1992b). It is the process of ice formation that needs to be controlled to prevent damage to the cells. During the process of cryopreservation, the temperature falls from 37°C to –196°C, and 95% of the cells' water can be lost. Concentrations of electrolytes increase both intracellularly and extracellularly, deforming cells, and ice can form inside cells disrupting the intracellular structures, rendering the cells non-viable.

There are two main methods of cryopreservation: freeze-thaw, either by slow or rapid cooling, and vitrification (Walcerz & Karow 1996).

5.1.2.1 Freeze-thaw – Slow cooling

The aim of this method is to freeze the extracellular fluid without ice formation inside the cells. During the process of slow cooling, large changes in the solution concentrations occur with the effect of causing mass transfer between the intracellular and extracellular compartments, which can damage the cell membranes; freezing occurs in the extracellular fluid before the intracellular fluid, due to the former compartment being relatively dilute. As ice forms, water is removed from the extracellular fluid, increasing its concentration and causing water to move out of the

cells due to the osmotic pressure gradient. Slow cooling allows water to diffuse out of the cells, thereby maintaining the osmotic equilibrium during freezing, but resulting in cell dehydration and shrinkage, which, if the volume of a cell shrinks by greater than 40%, can cause permanent cell damage. Therefore, the composition and temperature of these compartments must be carefully controlled throughout the process to avoid injury to the cells due to intracellular ice formation and solution affects. Cryoprotective agents (CPAs) and controlled cooling rates are used to achieve this. This slow cooling method with glycerol as CPA was used to cryopreserved the first cells (Polge et al. 1949).

5.1.2.2 Freeze-thaw – Rapid cooling

In contrast, if the cell suspension is cooled rapidly there is no time for the solute concentrations to cause an effect and instead ice forms uniformly. However, this method increases the likelihood of water remaining within the cells, as freezing occurs rapidly without time for diffusion from the intracellular to the extracellular space. Thus, the concentration of the intracellular fluid remains low, increasing the formation of intracellular ice and cell rupture.

5.1.2.3 Vitrification

Vitrification is the process by which liquids solidify, or vitrify, without crystallization during cooling. This is a less complicated method of cryopreservation, but more difficult to achieve. The transition from a disorganised liquid into an organised crystal, such as ice, requires the molecules to align, which takes time. If the temperature is lowered rapidly, there is no time for crystals to form, thereby preventing the formation of ice throughout the cell suspension when cooled to liquid nitrogen temperatures. Therefore, this method causes minimal disruption to cellular organisation, as the lack of ice in the system means that solute concentrations will remain constant; there will be no mass flow and no changes in cell volume.

However, to achieve this constantly, very high concentrations of CPAs are needed and, at these high concentrations, CPAs can have a toxic effect on cells. Therefore, the principal difficulty in designing vitrification procedures is finding a CPA that

permeates cell membranes in a mixture that is sufficiently concentrated to allow vitrification without being toxic.

5.1.2.4 Cryoprotective agents (CPAs)

CPAs are substances that protect cells against injury that result from the solidification of water and lipid during cryopreservation. They are water soluble, non-toxic to cells in lower concentrations and can be classified into two main types: those agents that permeate cells, including glycerol and dimethyl sulphoxide (DMSO), and those that do not, including starches and sugars.

The exact mechanism by which these agents work is not fully understood. The first type of CPAs are most commonly used and work by diffusing across the cell membranes, increasing the concentration and reducing the freezing point of the intracellular fluid, thereby reducing the osmotic gradient across the cell membrane which otherwise causes cell dehydration and shrinkage. The increased solutes in the intracellular fluid also help to reduce the freezing point of the intracellular fluid, thereby further reducing the likelihood of ice forming within cells. Intracellular CPA, in addition to reducing the osmotic pressure across the cell membrane, also diffuses into cells replacing the volume of water lost by dehydration. As CPAs diffuse across the cell membranes at a slower rate than water, slow cooling allows time for the equilibrium of the fluids and reduces cell rupture.

Other CPAs improve cryopreservation by remaining in the extracellular fluid, osmotically altering the compartments. This makes the extracellular fluid hypertonic relative to the intracellular concentration and therefore dehydrates the cells. The addition of CPAs to the extracellular fluid also reduces the freezing point of this fluid, allowing crystals to form slowly as the suspension is cooled. As the extracellular water freezes, the concentration of the fluid increases and so, draws more water out of the cells by osmosis. Severe cell shrinkage is therefore more likely to occur using CPA that remains outside the cells.

5.1.2.5 Cooling & Freezing

The cell membrane is an effective barrier to ice formation therefore, if the CPA has diffused into the cell without damage to the membrane, ice will be excluded from the cells as the temperature is reduced. Ice forms as the extracellular fluid is cooled, increasing the concentration of the compartment. If the temperature is lowered slowly there will be time for water to diffuse out of the cells, maintaining the osmotic equilibrium. However, if the cooling rate is too fast, water is unable to diffuse out of the cells, causing cells to be supercooled and increasing the probability of intracellular ice formation and cell death. It has been proposed that susceptible cells will not survive direct transfer from 37°C to zero and need to be cooled slowly 1°C/min (Morris et al. 1983).

This suggests that slow cooling increases cell viability, but experiments have shown that very slow cooling can also be damaging. Survival rates as a function of cooling produces a bell-shape curve, with 0.5 to 2.5°C/min as the optimum.

5.1.2.6 Resuscitation

From the low temperature of liquid nitrogen in which cells are stored, cells are warmed quickly to above melting point to minimise the damage due to recrystallisation of extracellular fluid and devitritication – the growth of intracellular ice crystals. By rewarming the cells rapidly the time needed for crystal formation is avoided. However, if warming is too fast large osmotic gradients can be generated that cause large amounts of water movement across the membrane, damaging the cell. The optimum rate of rewarming to balance the risk of cell damage has yet to be established and further research is needed.

CPA that has entered cells during their cooling also needs to be removed as, if it remains in the cells as the mixture thaws, the intracellular solute concentration will be greater than the extracellular, causing water to diffuse into the cells, resulting in swelling and cell rupture (the membrane is unable to stretch to any great degree). CPA is removed by the addition of warm medium in serial dilutions, allowing the CPA to diffuse out of the cells. Equilibration is necessary following each addition of medium, as CPA moves cross the cell membrane at a slower rate than water.

5.1.3 The method of cryopreservation used

Current cryopreservation processes cannot be applied universally to all cell types, as each cell type has an optimum-cooling rate defined by the permeability of the cell membrane to water. Furthermore, as the ability of cells to tolerate increasing concentrations of CPA varies, the highest concentration of CPA tolerated should be used to provide maximum protection without toxicity, although increasing concentrations require more complex methods of dilution on resuscitation for removal of CPA. Therefore, the method of cryopreservation used needs to be investigated for each cell type to determine protocols that allow maximum cell viability following resuscitation. This chapter studies the effect of cryopreservation on MSCs using two similar methods, with the only difference between them being the rate of cooling of the cells suspended in CPA. Comparing the specific effects of different types of cryopreservation is outside the remit of this chapter and requires further investigation.

In previous experiments, haematopoietic stem cells present within umbilical cord blood were first concentrated to reduce the volume that needs to be stored and then cryopreserved, with good cell recovery rates (Quillen & Berkman 1996). As MSCs are similarly present in very low density in bone marrow, in my study the fraction of marrow containing these cells will be separated using the Ficoll® separation method, as described in Chapter 2, prior to cryopreservation, also reducing the storage space required.

Furthermore, erythrocytes (red blood cells) present in bone marrow do not behave like mononucleated cells during cryopreservation and, following this process, if reimplanted into humans, can cause a toxic reaction proportional to the concentration of CPA used (Rowley SD 1992a). Although in my study, the number of erythrocytes that were cryopreserved was reduced by using Ficoll® separation, further means of removing erythrocytes may be necessary before the cells were implanted into patients to prevent such a toxic reaction. This could be achieved by culturing the resuscitated cells *in vitro*, as erythrocytes and cells containing CPAs do not survive in these conditions.

Due to the acknowledged low density of MSCs in bone marrow, to obtain a sufficient number of cells for tissue engineering applications, it would also be necessary to culture expand the MSCs. Thus, the bone marrow fraction, containing MSCs, would be resuscitated before the patient needed the engineered tissue-graft. Cells would be culture expanded and seeded onto the osteoconductive scaffold, where the cells would adhere and produce extracellular matrix. Following which the graft would be implanted.

A further advantage to cryopreservation storage of freshly isolated MSCs is that it provides a store of cells, if the primary culture becomes infected during expansion, preventing the need for a second bone marrow aspirate to be harvested in this situation. Therefore, in this chapter, a study was made of whether MSCs can be stored immediately after harvesting without loss of viability.

In this study, the method of cryopreservation involved the use of a CPA that diffuses into the cells to protect them during the freezing process. The vitrification process was not used in this study, as it requires high concentrations of CPA, which can be toxic to cells. Further, as CPA toxicity increases with time and increased temperature, in my method, exposure of the cells in the freezing mixture to room temperature was kept to a minimum.

As noted above, it is necessary to keep a balance in the slow cooling method, between cooling too slowly or too quickly. Although a longer cooling time reduces cell damage, this is in direct conflict with the need to limit cell exposure at higher temperatures to reduce CPA toxicity. As a result, two slightly different procedures of cooling MSCs were used in two experiments in this chapter. The first cooled the samples quickly to –70°C following the addition of CPA. Although this minimises the exposure time and reduces the toxic effects of CPA, it increases the risk of intracellular ice formation due to the speed of cooling. In the second method the cell mixture was cooled slowly allowing osmotic equilibration, which reduced the risk of ice forming within the cells, but increased the exposure time and potential for toxicity of CPA.

Lastly, DMSO was the CPA used in my study, as it has been found to be most effective, producing rapid diffusion into cells (Walcerz & Karow 1996). It is a by-

product of paper manufacturing and is a hydroscopic polar compound that is also used as a solvent for insecticides. On resuscitating the cells in this study, the cryovials were warmed in air rather than in a water bath, to allow warming without large osmotic shifts and crystal formation.

5.1.4 In this chapter

The aim of the studies in this chapter was to determine whether cryopreservation could be used to store bone marrow-derived MSCs before transplant and to determine whether this method of storage has any effect on proliferation rate and osteogenic potential of the MSCs. Cryopreservation was performed immediately after bone marrow aspiration and the results were compared with a control from each patient sample that was cultured from the time of harvest. After resuscitation the behaviour of the cell cultures *in vitro* was observed and compared against the control. Following this, serial proliferation rate after each of 4 passages was compared between cells that had been cryopreserved and the control. For MSCs to be useful for the tissue engineering of bone, the method of storage should not affect the differentiation ability of the cells into osteoblasts, thus this was also tested.

5.1.5 Hypothesis

Human MSCs can be cryopreserved and stored as a fresh bone marrow aspirate with no loss of cell proliferation or differentiation into osteoblasts. The three specific hypotheses tested in this chapter were:

1. Bone marrow aspirate fractions can be stored by cryopreservation and successfully resuscitated to produce viable MSCs to grow *in vitro*.
2. Cryopreservation does not affect the MSCs ability to differentiate into osteoblasts when stimulated in culture by OS, as compared to cells in standard culture.
3. Cryopreservation does not affect the proliferation rate of MSCs after resuscitation.

5.2 MATERIALS and METHODS

5.2.1 Materials

Cell culture materials as Chapter 2

Dimethyl sulphoxide (Sigma D2650)

Freezing container Nalgene (Sigma C1562)

Cryovials (Nunc 343958)

Isopropyl alcohol (BDH Chemicals)

ALP, osteopontin, osteocalcin and DNA assays as Chapter 2

Alamar blue assay as Chapter 3

5.2.2 Bone marrow aspiration and cryopreservation

Two experiments were conducted in this chapter, using different methods of cryopreservation, which varied the rate of slow cooling of the cell suspensions. For these experiments, bone marrow aspirates were taken from fifteen patients - ten aspirates were used for Experiment 1 and 5 for Experiment 2. In both cases, each patient's bone marrow aspirate was divided into two halves, from which the fraction of marrow containing MSCs was isolated by the process of Ficoll® gradient separation, using methods described in Chapter 2. Two cell pellets were thus generated from each aspirate. One pellet was cryopreserved, while the other was seeded in a culture flask as a control.

5.2.3 Experiment 1

5.2.3.1 Cryopreservation Method

5.2.3.1.1 Storage

From each of the 10 bone marrow aspirates, one of the cell pellets was resuspended in 1ml of freezing mixture (10% DMSO in foetal calf serum) using a 23G needle. The cell suspensions were then transferred to cryovials and placed in a –70°C freezer for 24 hours, following which the vials were transferred to liquid nitrogen for a further 7 days.

5.2.3.1.2 Resuscitation

The vials were removed from the liquid nitrogen and placed in the incubator at 37°C until the ice had almost thawed, at which point they were transferred to the laminar air-hood. An equal volume of 1ml of medium, warmed to 37°C, was initially added to each vial of cells and the suspensions were mixed through 23G needles. They were then left for 1 minute to allow temperature equilibration and this was done between each addition of medium. Following addition of the first ml of medium, the cell suspensions were transferred to a universal container, where 2ml and then 4ml of warm medium were added until a volume of 8ml was reached. The cell suspensions were centrifuged at 2000rpm for 5 minutes, resulting in the reformation of cell pellets. These were resuspended in 2ml of medium and transferred to culture flasks.

5.2.3.2 Cell culture

The primary cultures from each aspirate (the control) and the resuscitated cryopreserved MSCs were grown at 37°C and 5%CO_2 in monolayer until confluent. Initially, to assess the cryopreservation method of the bone marrow isolated cells, cells that had been resuscitated were observed under light microscopy and photographs were recorded.

To assess the effect of this method of cryopreservation, cells that had been cryopreserved and the control cells were cultured following the scheme detailed in figure 4.1a and 4.1b. The cells reached confluency at 5 days, at which point they were passaged, counted and seeded in well plates for biochemical and Alamar blue assays, to assess proliferation and osteoblastic differentiation (see figure 4.1). The remainder of the cells were then divided into 2 flasks, one continued to be cultured in standard medium and the other in OS.

5.2.3.3 Assessment of differentiation of MSCs into osteoblasts

5.2.3.3.1 ALP

The effect of cryopreservation on the potential differentiation of MSCs down the osteoblastic lineage was assessed, by culturing the cryopreserved and control

Figure 4.1a: The culture scheme followed for Experiment 1, by the control population of MSCs

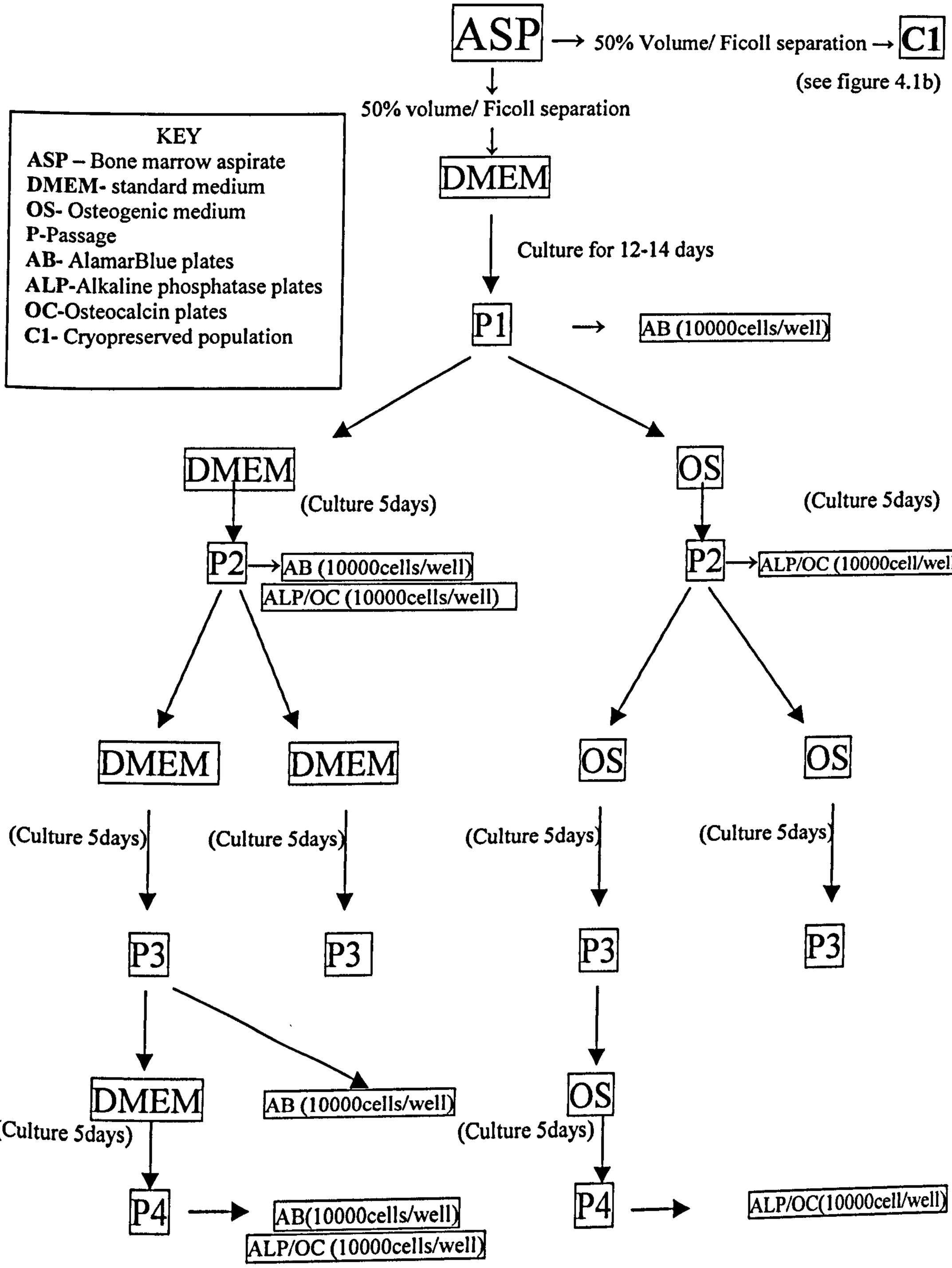
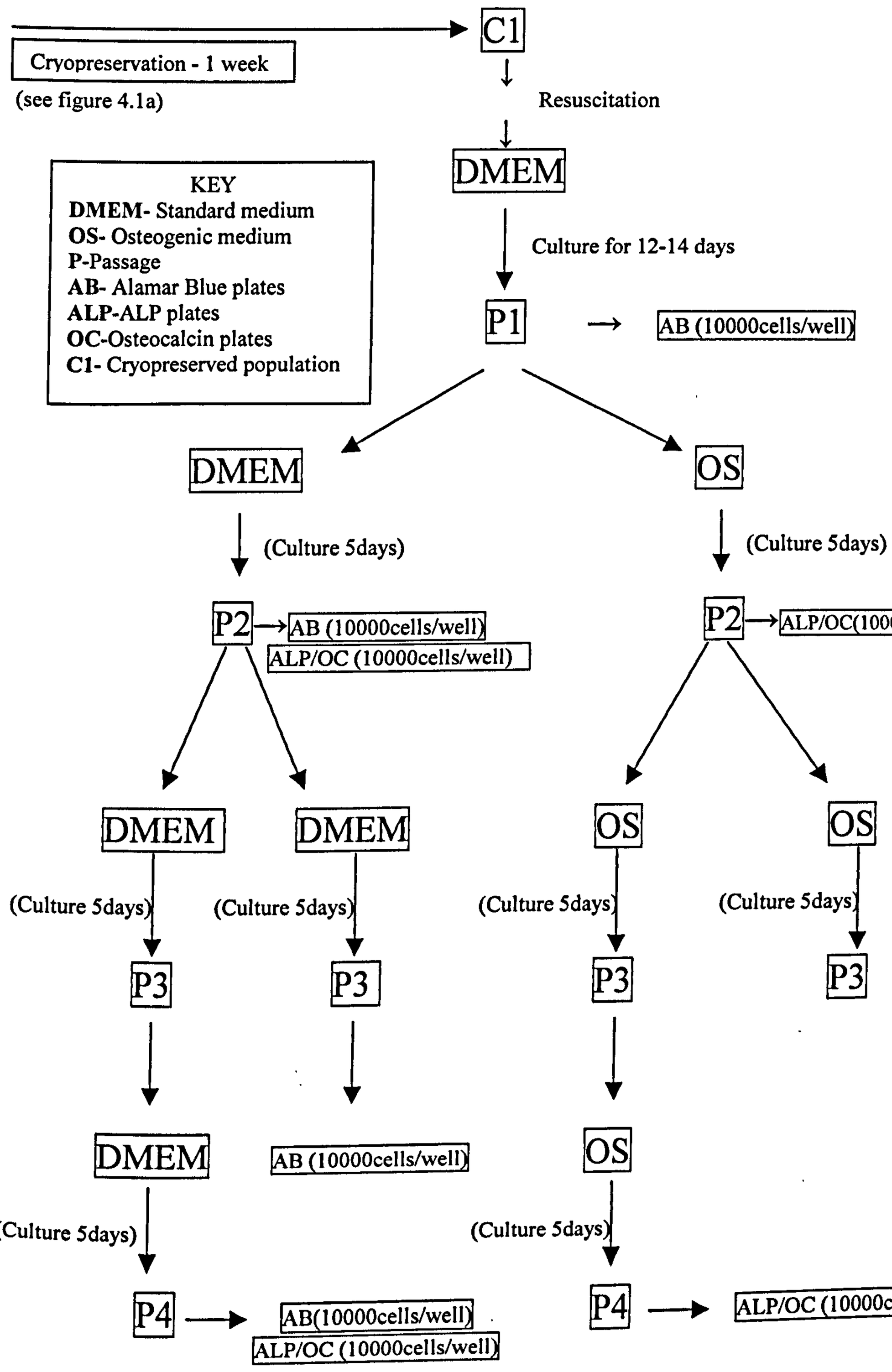


Figure 4.1b: The culture scheme followed for Experiment 1, by the cryopreserved population of MSCs



populations with OS as compare to standard medium and measuring the production of osteoblastic protein markers by each sample of cells.

In Experiment 1, 15 sets (5 patients, 3 samples for each) of 10,000 cells were cultured for each time point: 5 and 15 days following passage 2 (P2), and 5 days following P4, for both conditions: osteogenic or standard medium, for both populations: cryopreservation compared with the control (see figure 4.1). The ALP protein production divided by total DNA content for each sample was measured using assays described in Chapter 2.

5.2.3.3.2 Osteocalcin

The production of osteocalcin/DNA was measured on 10 sets (5 patients, 2 samples for each) of 10,000 cells cultured in either OS or standard medium for 15 days after P2. This was repeated for cells that had been cryopreserved, to assess the effect of cryopreservation on MSCs differentiation resulting from culture with OS (see figure 4.1). The assay used to measure osteocalcin was described in Chapter 2 Method.

5.2.4 Experiment 2

5.2.4.1 Cryopreservation method

5.2.4.1.1 Storage

For the second experiment the method of cryopreservation was revised, with the cell suspension in DMSO freezing mixture being cooled at a rate of -1°C/minute to –70°C. This was achieved by placing the cryovials in a Nalgene® freezing container containing isopropyl alcohol in the base, for 24 hours in the –70°C freezer before transfer to liquid nitrogen.

5.2.4.1.2 Resuscitation

After 7 days in liquid nitrogen cells were resuscitated using the same process for Experiment 1.

5.2.4.2 Cell culture

The primary cell aspirate and cryopreserved populations were cultured as for Experiment 1. Both populations of cells were culture expanded up to P3, following which MSCs were grown in well plates for biochemical assays. The number of cells used in each sample in Experiment 1 was small and on analysis of the results it was felt that this could explain the lack of osteocalcin detected so in Experiment 2, 200,000 cells were seeded per well.

5.2.4.3 Measures of differentiation of MSCs into osteoblasts

5.2.4.3.1 ALP

Ten samples (5 patients, 2 repeats for each) of 200,000 MSCs, from P3 were cultured for 7, 14 and 21 days in either OS or standard medium (see figure 4.2). Following which ALP/DNA content was measured, as in Experiment 1 and compared between the cryopreserved and control populations for each condition.

5.2.4.3.2 Osteopontin

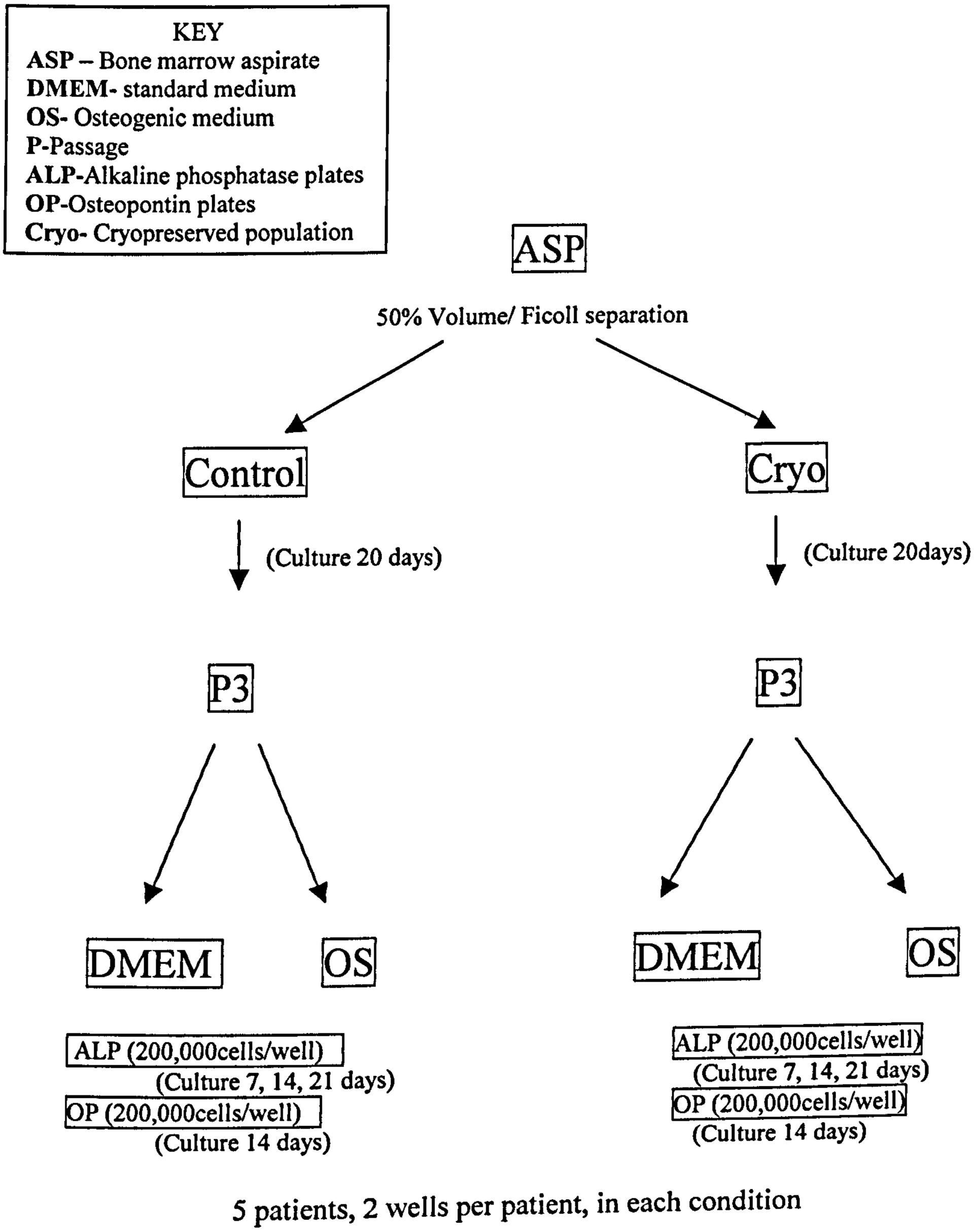
Osteopontin protein levels were measured after 14 days in OS compared with standard conditions, for both cryopreserved and control MSCs (see figure 4.2), using the method described in Chapter 2. A similar ten samples to those used in the ALP study were used (see 5.2.4.3.1).

5.2.5 Assessment of cellular proliferation rate

5.2.5.1 Alamar blue assay in Experiment 1

To assess quantitatively the proliferation rate of MSCs, Alamar blue assays were performed on cryopreserved and control cells in standard culture, after each passage until the fourth. Thus, for each of the 10 patients aspirates, 6 wells with 10,000 cells (total 60 samples) in each were cultured, as shown in figure 4.1. As Alamar blue is non-toxic to cells in culture, the assay was performed serially after days 1, 5 and 15 following each passage in Experiment 1 (for details of the Alamar blue assay see Chapter 2 Method).

Figure 4.2: The culture scheme followed for Experiment 2, by the control and cryopreserved population of MSCs.



5.2.5 Statistical tests

The results from MSCs that had been cryopreserved were compared with cells that had been cultured directly following aspiration. Statistical test were used to assess the distribution of the results and Kolmogorov-Smirnov and Shapiro-Wilk tests shows that the results did not follow a normal distribution. As a consequence, non-parametric statistical tests were used to analysis the data. These were performed to compare the effect of cryopreservation on osteoblastic protein production following the addition of OS to the culture and the proliferation rate of the cells as measured by Alamar blue.

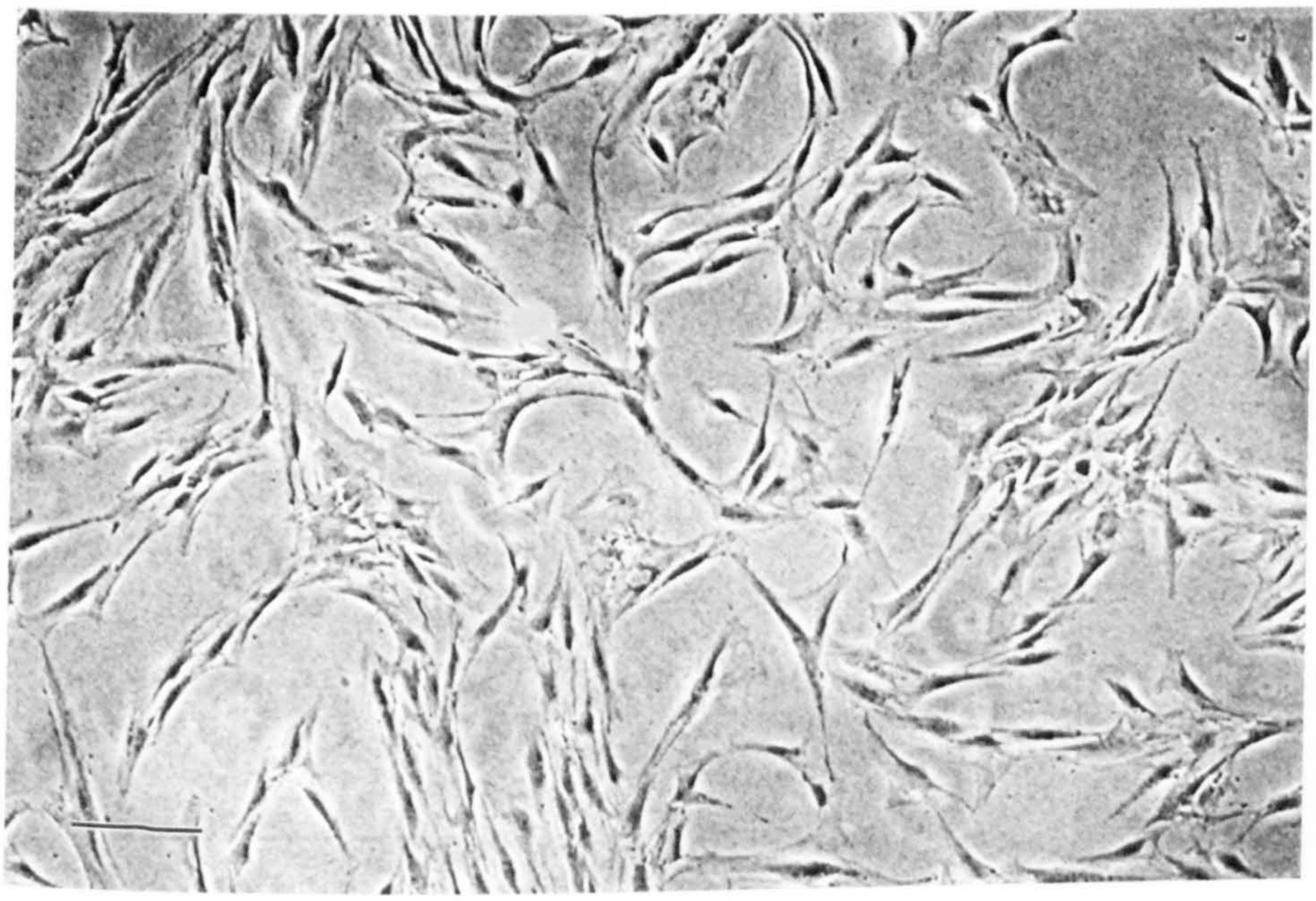
5.3 RESULTS

5.3.1 Assessment of Cryopreservation storage methods

5.3.1.1 Light microscopy observations

Both control and cryopreserved MSCs were observed to grow in culture, behaving in a similar manner. In primary culture the MSCs resembled spindle-shaped fibroblasts in morphology (see figure 4.3), formed colonies and became confluent after a similar time in culture, 12 – 14 days, irrespective of cryopreservation.

Figure 4. 3: Light microscopy pictures of MSCs from one of the control samples, after 14 days in culture, bar = 200μm.



5.3.2 Osteoblastic differentiation potential following cryopreservation

5.3.2.1 Light microscopy observations

Following the addition of OS a change in morphology was noted in both the cryopreserved and control cultures, with the cells becoming smaller and less spindle-like (see figure 4.4).

Figure 4. 4: Light microscopy pictures after MSCs had been cultured in OS for 7 days, following cryopreservation, bar =200μm.

5.3.3 Measurement of Osteogenic differentiation potential – Experiment 1

5.3.3.1 ALP protein production

For both the cryopreserved and control populations, ALP production was measured quantitatively and the amount produced by MSCs cultured in OS was compared with standard medium. As the results did not follow a normal distribution, non-parametric tests – Mann Whitney U were applied, unless otherwise stated, this was the case for all statistical comparisons in this chapter.

The production of ALP/DNA was significantly greater for MSCs cultured with OS compared with the control standard medium, for both the cryopreserved and control populations at each time point measured ($P<0.005$), (see figure 4.5).

The increase in ALP/DNA production for the cells cultured in OS in excess of in standard medium was calculated for each time point, by measuring the ALP/DNA for the MSCs grown in OS minus the ALP/DNA for the corresponding control. Hence, the effect of cryopreservation on the increased production of ALP by cells stimulated by OS could be assessed. There was not found to be a significant difference in the increase in ALP/DNA between MSCs that had been cryopreserved compared with the control ($P>0.05$), (see figure 4.6).

Figure 4. 5: A box plot showing the production of ALP/DNA for both cryopreserved and control cells, when MSCs were cultured in OS compared to standard medium (DM), *$P<0.005$ (^{o} outliers).

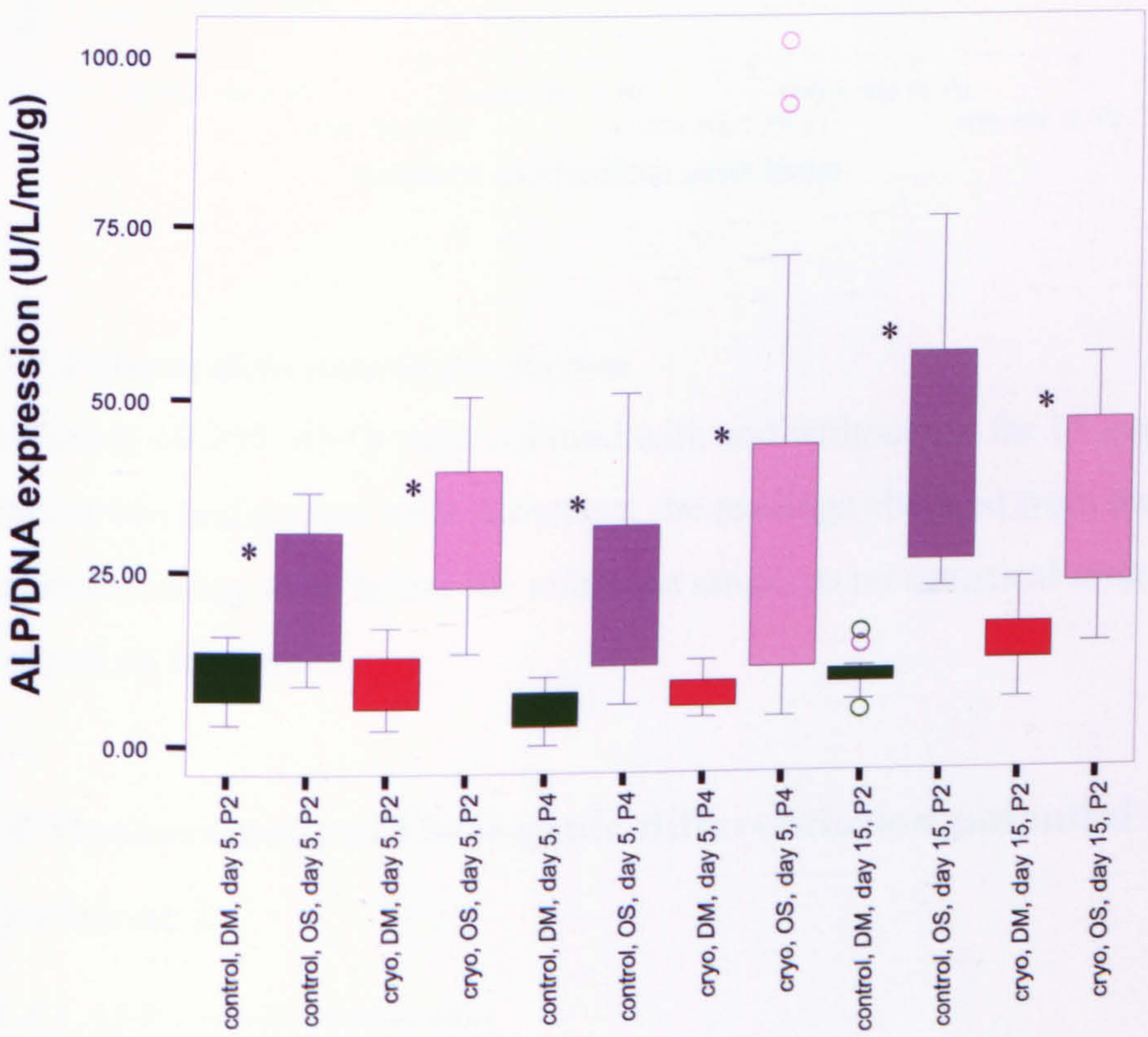
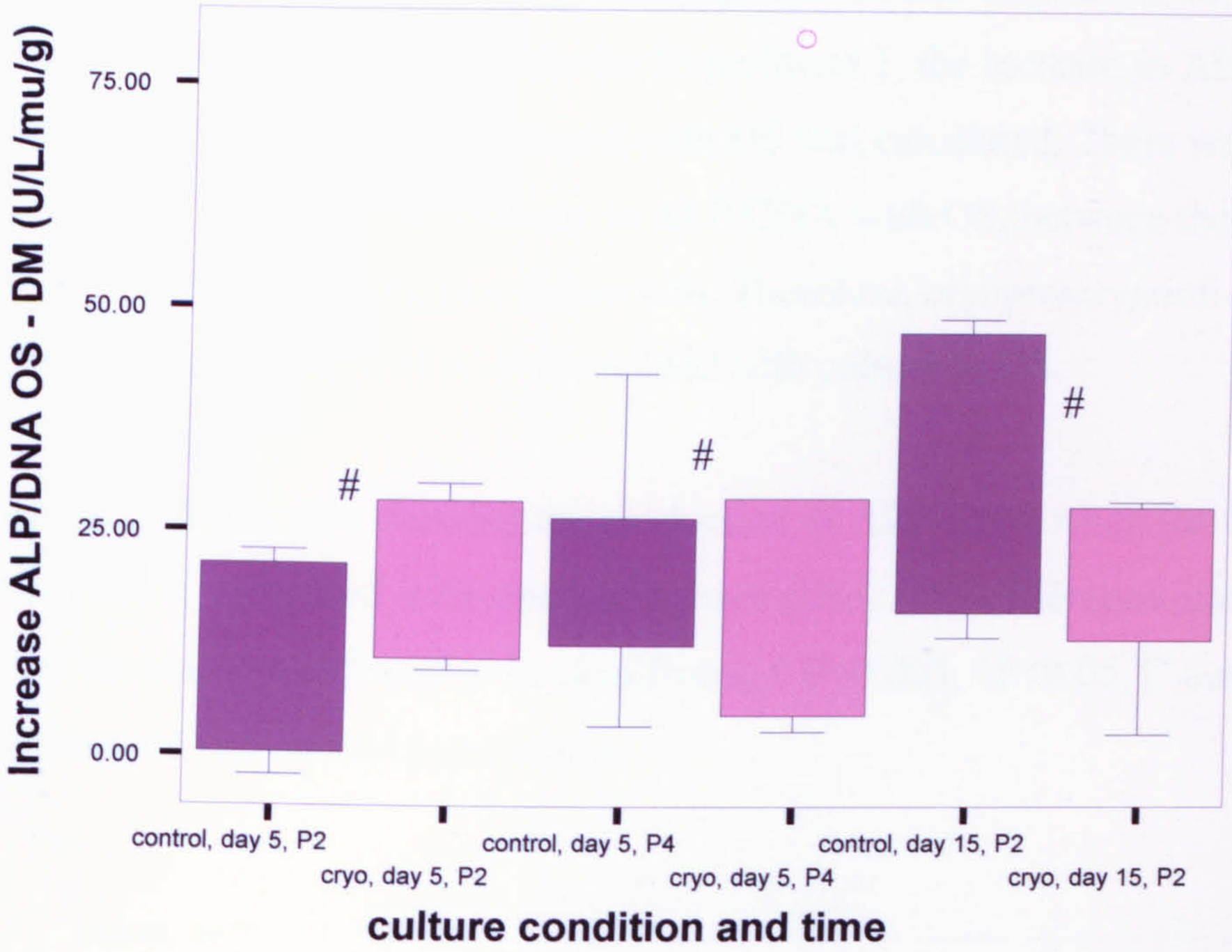


Figure 4. 6: Box plot showing the increase in ALP/DNA for cells cultured with OS above standard conditions (OS – DM), comparing between MSCs that have been cryopreserved and the control populations, # P>0.05, (° outlier).



5.3.3.2 Osteocalcin protein production

In this study 10,000 MSCs were cultured with and without OS for 15 days for both the cryopreserved and control cells. However, the readings obtained from the radioimmunoassay were below the reference range, so no statistical tests could be performed on the results.

5.3.4 Measurement of Osteogenic differentiation potential – Experiment 2

5.3.4.1 ALP protein production

In Experiment 2 a larger number of cells (200,000) were cultured for each sample, for the cryopreserved MSCs compared to the control, for different time periods, 7, 14 and 21 days, with and without OS. The ALP/DNA production increased when MSCs were cultured with OS compared with standard medium, for both the cryopreserved and control cells ($P<0.005$ for day 7 and 14 and $P<0.05$ for day 21 cryopreservation

group), (see figure 4.7). These results indicated that MSCs were producing more ALP when stimulated by OS, irrespective of whether they had been cryopreserved.

Figure 4.8 shows the calculated increase in ALP/DNA production when then cells were cultured with OS, comparing the cryopreserved and control MSCs, after each time period. Similarly to the results for Experiment 1, the increase in ALP/DNA production when MSCs were cultured with OS was calculated. There was no significant difference in the increase in ALP/DNA with OS, between the cryopreserved and control MSCs ($P>0.4$), (see figure 4.8). Therefore, cryopreservation does not effect the increased production of ALP associated with culture in OS.

Figure 4. 7: A box plot showing the production of ALP/DNA when the MSCs were cultured in OS compared with standard culture (DM). The difference after each time interval measured was found to be significant, * $P<0.005$, ^$P<0.05$, (^{o} outliers) for both cryopreserved and control populations.

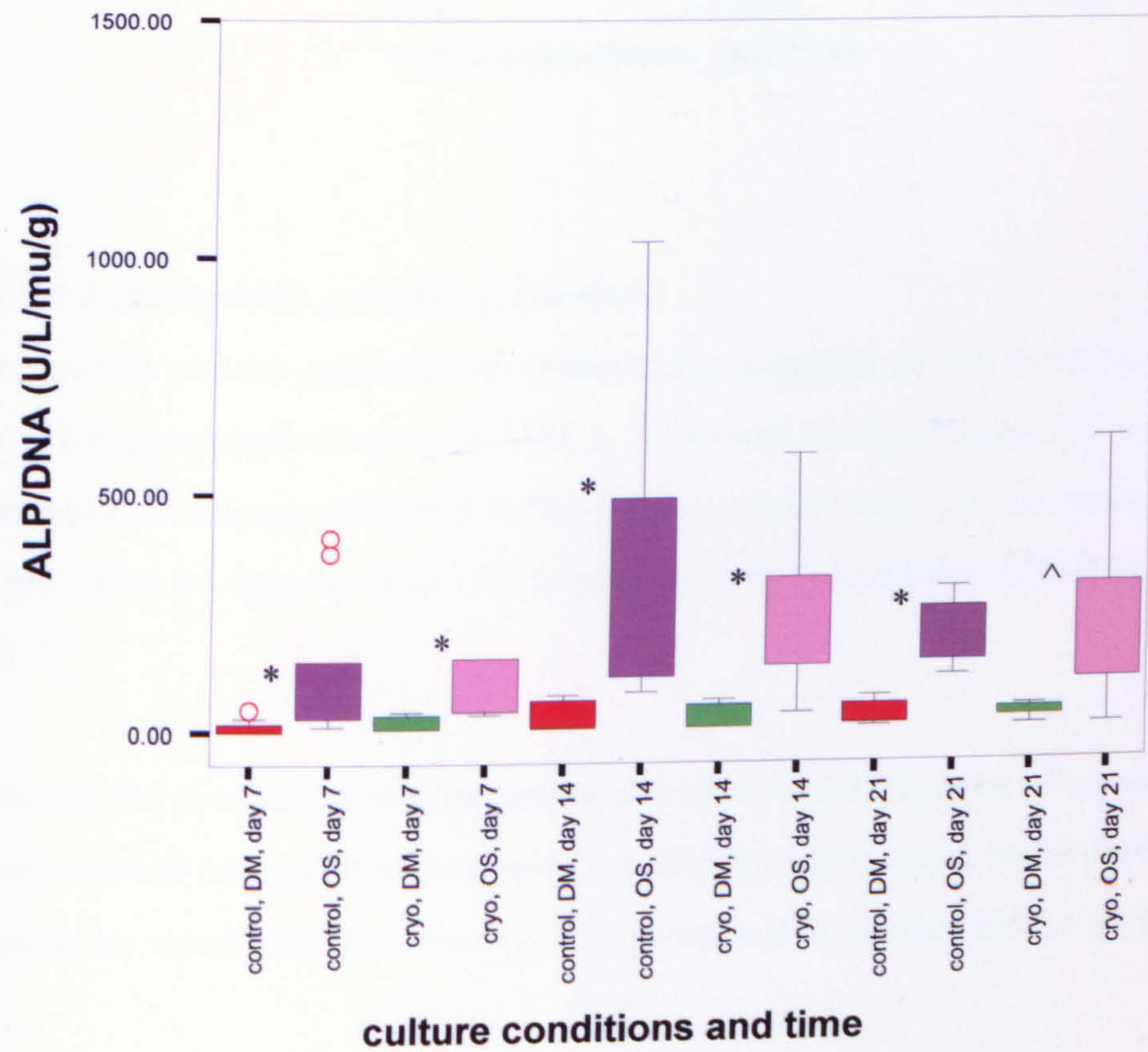
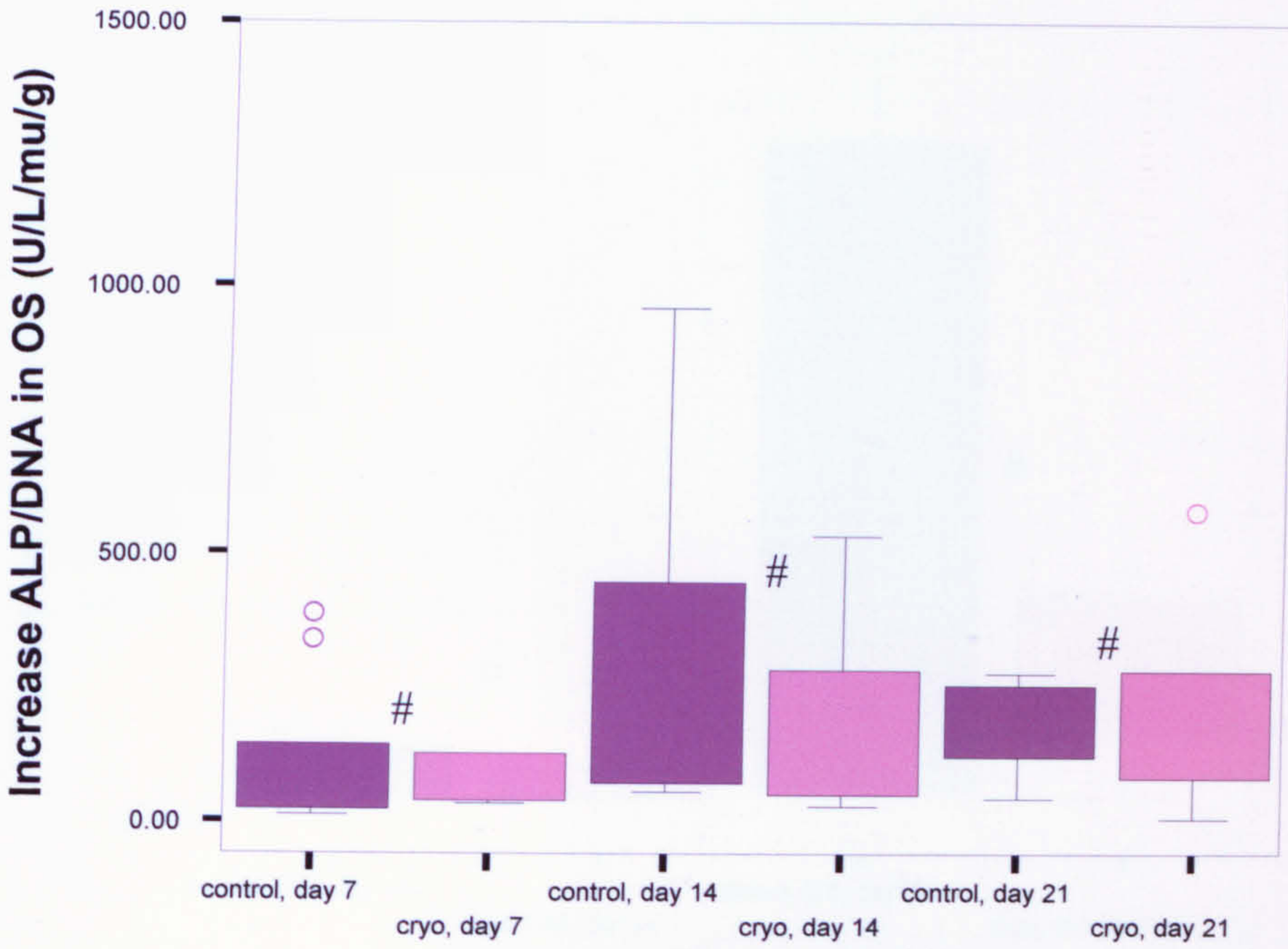


Figure 4. 8: Box plot showing the increase in ALP/DNA for cells cultured with OS above standard conditions, comparing cryopreserved cells with the control population # P>0.4, (° outliers).

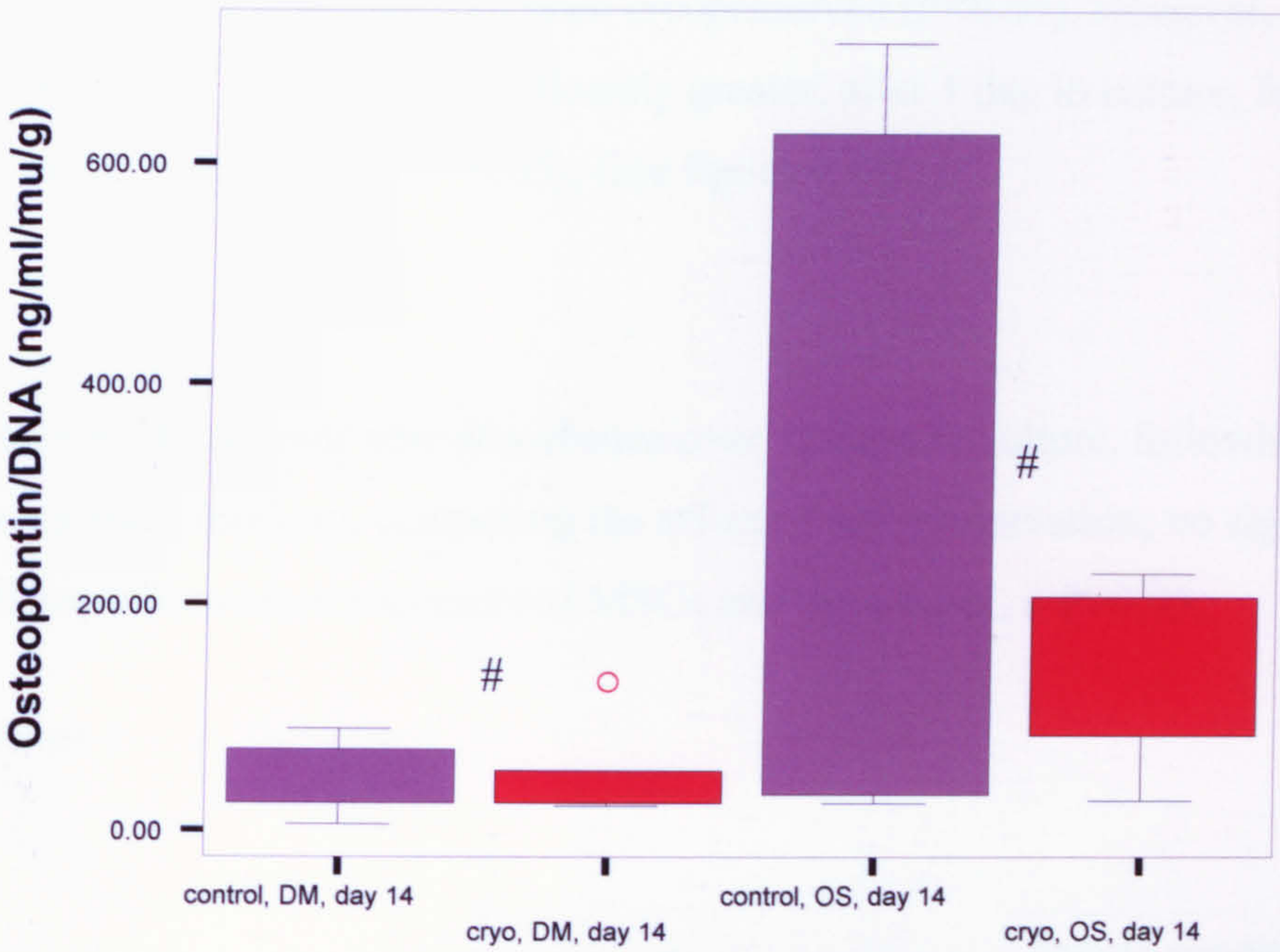


5.3.4.2 Osteopontin protein production

Osteopontin protein synthesis was measured as a marker of cell function to compare the effect of cryopreservation on MSCs. There was no significant difference in the production of osteopontin/DNA following cryopreservation as compared with the control, after 14 days in either OS or standard culture conditions (P>0.5), (see figure 4.9).

Although the production of osteopontin was observed to increase when the MSCs were cultured in OS compared with standard conditions for 14 days, the difference was not found to be significant, for either the cryopreserved or control MSCs (P<0.05).

Figure 4. 9: Box plot showing the production of osteopontin/DNA by MSCs comparing cryopreservation with the control; no significant difference between these two groups (# P>0.2), for either standard medium (DM) or OS, (° outlier).



5.3.5 Assessment of Proliferation rate – Experiment 1

5.3.5.1 Alamar blue assay results

The alamar blue absorbance increased over the 15 day time period for culture (see figure 4.10). Therefore, each sample of cells increased in proliferative activity over the culture period.

Figure 4.10 shows the comparative absorbance of alamar blue between the cryopreserved and control MSCs over 15 days in standard culture conditions, following passages 1-4 (P1-4). There was no significant difference in alamar blue

absorbance between the cryopreserved and control MSCs, after 15 days in culture following each passage tested (P>0.05). In addition to this, there was no significant difference after 5 days in culture following P1, 2 and 4 (P>0.05).

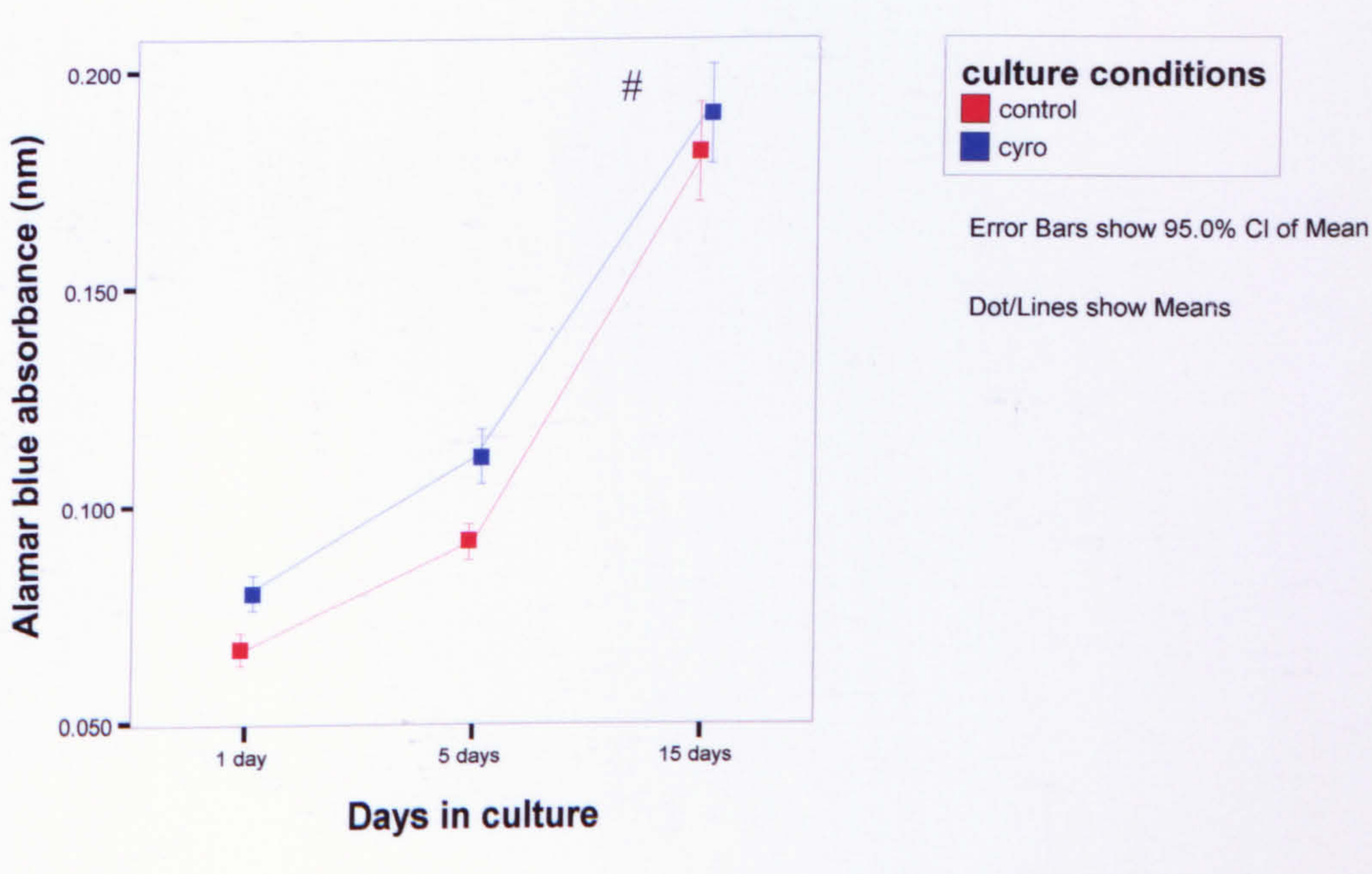
After 1 day in culture following P1, the absorbance of alamar blue was significantly greater for MSCs that had not been cryopreserved (P<0.05). However, following P2, 3, and 4, the absorption was significantly greater, after 1 day in culture, for the MSCs that had been cryopreserved (P<0.05), (see figure 4.10).

Figure 4. 10: Alamar blue absorbance over 15 days in culture, following passages 1 – 4 in standard medium, comparing the effect of cryopreservation; no significant difference between cryopreserved MSCs and the control, # P>0.05.

Passage 1

Passage 2

Passage 3



Passage 4


culture conditions
control
cyro
Error Bars show 95.0% CI of Mean
Dot/Lines show Means
Alamar blue absorbance (nm)
0.200
0.160
0.120
0.080
#
#
1 day
5 days
15 days
Days in culture

5.4 DISCUSSION

For MSCs to be used for tissue engineering applications, a reliable source of bone marrow-isolated cells is needed. Cryopreservation was tested in this chapter as a method of storing MSCs in liquid nitrogen for 7 days. If this method was to be used clinically for cell storage, especially if allogenic cells could be used clinically and a bank of MSCs created, the cells would need to be kept for longer than 7 days prior to use. However, it is the processes of freezing and resuscitating cells that put the cells at risk of damage. While cells are in liquid nitrogen storage they are stable and the only risk is to the DNA from background radiation, the effect of which occurs over a number of years. Therefore, similar results would be expected if the MSCs were stored by cryopreservation for months rather than days.

5.4.1 Effect of cryopreservation on observations of cells in culture

The first hypothesis in this chapter stated that, following isolation from bone marrow, MSCs could be stored by cryopreservation and successfully resuscitated. This was indicated to be the case, as every cryopreserved aspirate was resuscitated successfully and grown in cell culture.

The cells in this study were observed to behave similarly in culture to those described in the literature (Friedenstein et al. 1970; Lazarus et al. 1995). Under light microscopy, each MSC sample was observed to have followed an identical pattern irrespective of cryopreservation, whereby MSCs in both sets of primary cultures formed colonies, as described in Chapter 2. It was this phenomenon, noted first by Friedenstein that caused him to name these cells as a separate cell type, 'Colony Forming Units- Fibroblastic', (Friedenstein 1976) and indicates the stem cell nature of MSCs (see Chapter 1). In addition, both populations of cells grew at similar rates becoming confluent after an equal time period in culture following each passage.

Although, as stated above, every cell sample was successfully resuscitated following cryopreservation, it was not possible to measure the percentage of cell survival, as this would require the number of viable MSCs to be counted and compared, before and after cryopreservation. While Ficoll separation reduced the volume of cells other than MSCs present in the marrow, it does not remove all the erythrocytes. As a result of

this, a relatively large numbers of erythrocytes obscured the MSCs when the marrow fraction was placed on a haemocytometer and consequently, MSC cell counts could not be reliably obtained.

Therefore, in order to measure accurately the percentage of viable MSCs, before and after the cryopreservation process, a specific cell marker would need to be used to count functioning MSCs. Stro-1 is one such marker that could be used in combination with a cell sorting method (Simmons & Torok-Storb 1991).

Alternatively, changes in cells viability following cryopreservation can be measured by observation of changes in cell function, such as protein production or proliferation rates. This measurement is more relevant to the potential clinical use of MSCs, where the cells need to maintain their ability to differentiate into osteoblasts. MSCs must also maintain their capacity for self-renewal to replace cell loss due to cryopreservation, compensating for this when the cells are expanded in culture after resuscitation and seeded onto a scaffold for tissue engineering uses. Therefore, differentiation into osteoblasts and proliferation potential were also measured in this study.

5.4.2 Conclusions from assessment of osteoblastic potential following cryopreservation

5.4.2.1 Light microscopy

Irrespective of cryopreservation, the morphology of the MSCs cultured with OS was observed to change and become consistent with the shape of osteoblasts (Aubin & Liu 1996; Holtrop 1990). This change in cell shape and size was not delayed for the cells that had been cryopreserved when compared with the control. Therefore, these observations were evidence that the process of cryopreservation did not effect osteoblastic differentiation of MSCs. However, morphology alone is a relatively poor marker of cell type and other markers of osteoblasts were measured for both populations.

5.4.2.2 ALP

ALP protein levels have been used previously in my thesis as a quantitative measure of osteoblastic differentiation (see Chapter 2). There was found to be a significant increase in ALP protein synthesis by MSCs cultured in OS compared with standard medium for all cryopreserved and control MSCs ($P<0.005$), which is consistent with the findings in Chapter 2. This was found for both Experiments 1 and 2, where slightly different cryopreservation methods had been used and the number of cells seeded and culture periods were varied. Therefore, cryopreservation does not have a significant affect on the production of ALP when MSCs are stimulated by OS.

In Experiment 1, the effect of osteogenic culture conditions was also assessed after passage 2 and 4, and a significant increase in ALP was noted on addition of OS compared with standard culture, irrespective of passage number or cryopreservation. Hence, after both passages 2 and 4 MSCs maintain their osteogenic potential, as measured by ALP, regardless of this method of slow cooling cryopreservation.

Furthermore, in Experiment 2, when a different cooling method was used and the temperature was reduced by 1°C/minute prior to cryopreservation, the same relationship was found, whereby ALP levels increased significantly when the MSCs were cultured in OS over the 15-day culture period, for both the cryopreserved and the control MSCs.

For both cryopreservation methods, the increase in ALP synthesis when MSCs were cultured in OS over the matched standard medium control was calulated. The increase in ALP indicates osteoblastic differentiation and was used to assess the effect of cryopreservation on the ability of MSCs to differentiate into osteoblasts. There was not found to be any significant difference in the increase in ALP when MSCs had been cryopreserved prior to culture (using either method from Experiments 1 or 2), indicating the same potential for osteoblastic differentiation.

5.4.2.3 Osteopontin

Osteopontin is a protein produced by osteoblasts during bone matrix deposition (Nakase et al. 1994), (see Chapters 1 and 2). Therefore, the synthesis of this protein

was measured to assess the effect of cryopreservation on the cellular function of MSCs. After 14 days, there was not found to be any significant difference in the expression of osteopontin between the MSCs that had been cryopreserved and the control population, cultured in either OS or standard conditions ($P>0.5$).

However, there was not found to be a significant increase in the production of osteopontin when the MSCs were cultured with OS as compared with standard conditions, for either the cryopreserved or control MSCs. In comparison, it was reported in Chapter 2 that, after 14 days in OS culture, a significant increase in osteopontin production was seen ($P<0.005$). Conversely, in that study a larger cell seeding density of 250,000 rather than 200,000 cells was used. The larger cell number would have caused the cells to become confluent more rapidly, which may have allowed earlier deposition of extracellular matrix, resulting in observed greater osteopontin production.

In Chapter 3, osteopontin production was measured after MSCs had been cultured for 14 and 28 days on HA. By the 28-day point, the osteopontin measurements were significantly greater than at the earlier point ($P<0.05$). Therefore, further to the experiments performed in this chapter, a larger number of cells would need to be cultured over a longer time in order to allow OS to stimulate MSCs to differentiate sufficiently to produce significantly greater amounts of osteopontin.

Nevertheless, for the culture conditions used in this chapter, it was observed that cryopreservation had no significant effect on the function of the cells measured by production of osteopontin, thus supporting the hypothesis that cryopreservation does not affect the MSCs ability to differentiate into osteoblasts *in vitro*.

5.4.2.4 Osteocalcin

Osteocalcin is an extracellular protein involved in binding calcium to the extracellular matrix during the mineralisation phase, and is therefore a marker of mature osteoblasts (see Chapters 1 and 2). In this study, 10,000 MSCs were cultured with and without OS for 15 days.

The small number of cells seeded and short culture period, compared with Chapter 2, where osteocalcin was found to be produced after culturing 250,000 MSCs in OS for 28 days, may explain why the osteocalcin levels were below the reference range in this study. To investigate this further, the experiment would need to be repeated using a greater seeding number of cells and longer culture period.

5.4.3 Conclusions from cellular proliferation rate assessment following cryopreservation

5.4.3.1 Alamar blue

The effect of cryopreservation on the proliferation rate of the MSCs was assessed using Alamar blue assays. Alamar blue is an indictor of cellular redox state, as metabolic activity of cells in culture causes the dye to change colour, the degree of which can be measured, equating to the proliferation rate of the cells (see Chapter 3 Discussion).

In this study, the absorbance of Alamar blue was measured for both the cryopreserved and control MSCs on 6 wells from each of 10 patients' samples, resulting in a total of 60 samples for each 15 day period of culture, following passages 1 to 4. In all samples, Alamar blue absorbance increased over 15 days, indicating that MSCs from both populations continued to proliferate over the culture period.

Although after day 1 following the first passage the Alamar readings were significantly greater for the MSCs that had not been cryopreserved ($P<0.05$), there was no significant difference in absorbance between the populations after day 5. It has been shown previously that cryopreservation had a negative effect on proliferation of chondrocytes until day 7 (Rendal-Vazquez et al. 2001). As it was not possible to count the number of resuscitated MSCs using a haemocytometer (see above), the Alamar blue assay could not be performed until after the first passage when comparative samples could be assembled. However, it seems that the initial period of relative inactivity of the cryopreserved MSCs was brief.

In contrast, subsequent to each passage 2 and 4, it was observed that, after 1 day in culture, the Alamar absorbance was significantly greater for MSCs that had been

cryopreserved. It has been shown that cryo-resuscitation can induce a stress response in fibroblasts that can trigger a compensatory release of growth factors (Liu et al. 2000). Such a response by cells to release growth factors may explain an increase in proliferation within the first few days following cryo-resuscitation of MSCs, but for the difference to have persisted over 4 passages modification would need to be inherited. In bovine retinal pigment epithelial cells, cryopreservation has been found to alter DNA (Basu et al. 1983), whereby the genotype of the cell could be changed. However, an increase in Alamar absorbance was not observed after passage 1 and did not continue after day 1 following each passage 2 and 4, and therefore is less likely due to a change in the cells as a result of cryopreservation and more likely to have occurred as a result of the passage itself or an unseen variation in the method used to assemble the cells for the experiment. Although, to clarify these observed changes further research comparing gene expression following cryopreservation compared with a control is necessary.

In my study, cryopreservation did not reduce the proliferation rate of MSCs after 5 days in culture following passage 1, 2 and 4, as measured by Alamar blue.

Chapter 6

Discussion

6.1 DISCUSSION

6.1.1 Conclusions from this thesis

In my thesis the isolation of MSCs from human bone marrow aspirates and their suitability for use in tissue engineering of bone was investigated. In order for these cells to be useful, their potential for osteoblastic differentiation needed to be demonstrated. It was suggested that HA would encourage such differentiation (Furlong & Osborn 1991; Yoshikawa et al. 2000), (see Chapter 3 Introduction) and thus MSCs were cultured on porous HA scaffold. Having confirmed that the cells indeed differentiated down the osteoblastic lineage, this capability was tested further in my novel bioreactor and storage methods for the cells were also investigated.

In Chapter 2 it was shown that a population of cells could be isolated from human bone marrow aspirates and grown in culture. Stro-1 positively identified the marrow-isolated cells and its expression was observed to decrease as the cells were stimulated to differentiate into osteoblasts, indicating that it was a primitive marker. Although Stro-1 has been suggested as a reliable marker of MSCs (Gronthos et al. 1994; Simmons & Torok-Storb 1991), it should be acknowledged that there are difficulties in finding consistent markers for stem cells due to the cells changing phenotype, in response to their environment.

It was also shown, in Chapter 2, that the isolated cells could be stimulated to differentiate into osteoblasts, chondrocytes and adipocytes, thus confirming their multipotency. However, as histological stains were used to identify the differentiated chondrocytes and adipocytes, this only allowed observational identification. Furthermore, the substance stained to identify chondrocytic cells was organised collagen extracellular matrix, which is not totally specific to collagen type II or to this cell type. However, as my thesis involved the tissue engineering of bone, the occurrence of osteoblastic differentiation was clarified further using additional phenotypic markers.

It was then shown that marrow-isolated cells produced significantly more ALP and osteopontin when cultured with OS. These proteins are known to be produced by

osteoblasts, but production is not specific to this cell type. Osteocalcin is specifically produced by osteoblasts and this was only produced when the MSCs were cultured with OS, thus confirming osteoblastic differentiation.

The small number of colonies that were cultured from each aspirate confirmed the low density of MSCs within the bone marrow. Large cell numbers are required for tissue engineering, so it was important to establish the capacity for MSCs to proliferate.

Cell proliferation was assessed using several methods, in my studies. DNA levels within samples were measured using Hoechst assay and, as the amount of DNA within a cell is constant, this was used to assess cell number. Although the assay does not distinguish between DNA from alive and dead cells, debris from dead cells would be removed with cell washes. Alamar blue assays were also used as a measure of metabolic activity of cells in culture over time, thus giving an indication of proliferation over a culture period (Ahmed et al. 1994; Nakayama et al. 1997). A simple method of assessing cell proliferation is to count the number of cells and this was done to assess growth on HA scaffolds. However, small errors in counting when multiplied can have a large effect on the results so, although this is the most direct measurement of proliferation it was not applied to other parts of my study.

In order to generate tissue-engineered bone for implantation into patients, cells that are committed to the osteoblastic lineage need to be grown on a substrate. In Chapter 3 the tissue engineering of bone was explored using marrow-isolated MSCs, as these cells can be differentiated into osteoblasts as demonstrated in Chapter 2. As HA is the major constituent of inorganic bone, an HA surface was investigated as a substrate on which to grow MSCs. It was found that MSCs grown on this surface produced significantly greater amounts of ALP and osteopontin; both indicators of cell differentiation down the osteoblastic lineage. Additionally, the cells produced osteocalcin, indicating that MSCs differentiated into osteoblasts. This differentiation occurred without the osteogenic stimulants that were needed in monolayer as shown in Chapter 2.

Various factors may explain my findings of osteoblastic differentiation of MSCs on HA. HA promotes the formation of an apatite layer on its surface, as a result of HA dissolution. Thus, as the extracellular matrix produced by osteoblasts is mineralised to

produce an apatite, it is likely that the extracellular matrix proteins produced by the differentiating cells attach to HA. Secondly, when HA alone is implanted at extra-skeletal sites *in vivo*, bone has been noted to form (Ripamonti 1996; Yamasaki & Saki 1992; Yuan et al. 1999), which suggests that HA may be osteoinductive when influenced by growth factors. Furthermore, HA with a rough surface has been shown to promote bone formation *in vivo* (Yuan et al. 1999). These factors may have stimulated the differentiation of MSCs into osteoblasts. The effect of HA is seen in patients as bone binds directly onto HA coated prostheses (Furlong & Osborn 1991; Okumura et al. 1997).

A 3-d structure is needed to construct a suitable graft for tissue engineering and as HA was shown to stimulate osteoblastic differentiation, a porous HA scaffold was investigated. Under SEM, MSCs were shown to cover the surfaces of the HA scaffold, attaching and proliferating on the surface. Evidence for MSCs binding to the HA was observed by the large numbers of cell processes attaching the MSCs to HA, seen under SEM and TEM in Chapter 3. The production of osteoblastic proteins, such as osteopontin, osteocalcin and ALP, by the MSCs cultured on HA scaffolds increased over the 15-day culture period indicating differentiation into osteoblasts. The micro-pores on the scaffolds' surface measured approximately 10μm, which is similar in size to canuliculi-lacunar structure within cortical bone (Qin et al. 1999). This may imitate the physiological environment and act as a further trigger for the attachment and differentiation of MSCs, although further work is needed to confirm this.

Investigations of cells cultured *in vitro* have traditionally used a 2-d environment. In these conditions the cells attach themselves and orientate in one direction on the culture surface. However, this is artificial when compared with the growth of cells *in vivo*. For example in bone, osteocytes are attached to other cells by long processes and are surrounded by extracellular matrix. The replacement of diseased tissue requires a biological structure similar to that of the original. Therefore one of the goals of tissue engineering is the formation of 3-d cell constructs.

Therefore, to tissue-engineer a bone graft for clinical use, the cells need to penetrate through the scaffold growing in an orientated manner on every surface, thereby

forming a 3-d construct like cells within the Haversian system of cortical bone. Furthermore, as a large constituent of bone is extracellular matrix, the cells need to produce this. In the design of my novel bioreactor the flow of fluid through living bone was simulated through the HA scaffold. This was found to increase cell penetration, measured by cell counts, differentiation tested by osteoblastic protein levels and extracellular matrix production, assessed by pro-collagen assays and TEM. The use of my bioreactor resulted in constructs that resembled bone tissue, where bone cells penetrated through the porous scaffold attaching to all surfaces and produced extracellular matrix.

HA is brittle and does not possess the same mechanical strength as bone and therefore, cannot be used for load-bearing support. However, the strength of HA grafts has been shown to increase once bone starts to grow into it (Ohgushi et al. 1989a). Therefore the strength of the tissue-engineered construct may be greater than HA alone, as a result of extracellular matrix formation.

The same methods of assessing cell proliferation and osteoblastic differentiation were used to determine whether cryopreservation affected the proliferation or differentiation capacity of MSCs. Although, the limitations of these assays, as discussed above, would also apply to these results, nonetheless it was shown that this means of storing freshly isolated MSCs can be used for cells prior to use in the tissue engineering of bone without affecting the abilities of MSCs.

In conclusion, MSCs can be successfully isolated from human bone marrow aspirates, which can be easily harvested from patients, and cultured for use in the tissue engineering of bone. HA has been determined as a suitable surface on which to culture MSCs as it stimulates differentiation into osteoblasts. My novel bioreactor enhances the tissue engineering of an osteogenic construct that could be implanted into patients to heal bone defects. Furthermore, as it was shown that cryopreservation does not affect MSCs potential for tissue engineering, this can be used to store cells prior to implantation. Although this research proves the concept, further testing in an animal model is necessary before clinical trials could be started.

6.1.2 Clinical relevance of the studies

From the *in vitro* experiments in my thesis, it can be concluded that MSCs can be used to construct grafts for potential use in tissue engineering bone defects. As detailed in Chapter 1, bone defects that are greater than a critical size will not heal. Such defects can be caused by fractures, excision of bone tumours and revision of joint replacements. These clinical situations are currently treated with either autologous or allogenic bone graft, in addition to synthetic substances, but these treatments are associated with various difficulties (see Chapter 1). Therefore, if my osteoinductive HA-MSC constructs were implanted into bone defect *in vivo*, bony healing would be expected. A further clinical use of my tissue-engineered bone would be in joint fusions, for example spinal fusions, where current practice uses a mixture of autologous and allogenic bone graft.

For bone marrow isolated MSCs to be used in patients in the way described above, a consistently reliable method of storage is needed for the cells, as the time between harvest and transplant of autologous cells may vary. Therefore, bone marrow could be harvested at the time of the primary operation, for example the debridement of osteomyelitic bone or compound fracture. The MSCs would be isolated and stored, before they were needed for clinical implantation at the second stage operation.

Cryopreservation has been identified as useful for the storage of biological material, as it is not associated with the risks of maintaining cells in culture, including infection; it also reduces the time and cost involved. In Chapter 5 it was confirmed that cryopreservation of MSCs freshly isolated from bone marrow did not alter the function of the cells. Thereby, for autologous MSCs to be used in clinical cases, once the time frame for implantation of the construct is determined, cells could be resuscitated and cultured on an appropriate scaffold. This method of storing the freshly isolated MSCs results in one culture process, reducing the risks associated with it. Continued storage by cryopreservation of half the harvested MSCs would also provide the security of a further sample if the primary culture becomes contaminated, removing the need for obtaining a second marrow aspirate. Patients could also have bone marrow isolated

MSCs cryopreserved for use in later life, for example possibly for the treatment of osteoporosis.

Furthermore, if allogenic MSCs were to be used for tissue engineering, a storage method to provide a reservoir of cells would be necessary. Therefore, a bank of cryopreserved MSCs could be created, from which cells could be taken and implanted to treat connective tissue defects. However, the implantation of allogenic cells into a patient carries the risk of host immune hypersensitivity reaction. To reduce the incidence of this immune reaction, the cells could be Human Leucocyte Antigen (HLA) matched, as applied to organ donation. However, it has been shown that a Dog Leucocyte Antigen (DLA) mismatch goes not affect the healing of a segmental bone defect in canines, with MSCs still improving the rate of bone healing (Livingston et al. 2001). If this is also the case for humans, it increases the potential clinical use of MSCs for the treatment of bone defects. Therefore, the immune reaction generated by allogenic human MSC needed to be investigated further.

6.1.3 Further work

For MSCs to be reintroduced into humans for tissue engineering, concerns may be raised about the theoretical transmission of prions from the foetal calf serum (FCS) used in culture medium. It has been shown that FCS is necessary for the initial adhesion of rat MSCs in primary culture, but following passage 1 this is no longer necessary for cell growth (Lennon et al. 1995). Therefore, to reduce the possibility of prion transmission from the serum into the cells, culture of MSCs without the use of FSC needs to be investigated. It may be possible to use small amount of autologous serum initially, separated from the patient's blood, thus avoiding the need for FCS.

As the HA surface used in this thesis was shown to stimulate the differentiation of MSCs into osteoblasts, HA with a similar macro and micro-porous surface could be used to coat prostheses. Further, autologous MSCs could be cultured on the surface of prostheses and following implantation the resulting bony ingrowth studied.

Use of the novel bioreactor also has massive potential for tissue engineering as it promotes 3-d growth of cells through a scaffold. Firstly, the effect of varying the flow

rate of medium through the system on the proliferation and differentiation of MSCs could be examined. The growth of MSCs on different scaffolds could be tested, for example, allograft or titanium could be investigated for increased osteogenesis. Furthermore, the penetration of cells through viscous substrates such as collagen or fibrin could be investigated will the aim of creating a glue-like substance that could be used in fracture healing. As the increased supply of nutrients was able to support a greater cell proliferation, further research would also include using a lower initial cell seeding density, thereby reducing the length of time in cell culture prior to growing MSCs on a scaffold and the overall time necessary to tissue engineer bone constructs. Additional bioreactor chambers set up in parallel within the system could be used to culture larger amounts of cells-scaffold constructs.

Further to being used for culturing osteoblasts from MSCs for the production of tissue-engineered bone, the principles of this bioreactor could be applied to other tissues. By varying the flow rate and scaffold on which the MSCs are cultured, different types of connective tissue could be engineered. For example, MSCs on an alginate substrate perfused with a lower tension of oxygen for the tissue engineering of cartilage or the differentiation of MSCs into fibroblasts when cultured on sheets of collagen for the tissue engineering of skin, could be investigated.

Lastly, cryopreservation has been shown to be an effective method of storing Dermograft®, which is a preparation of fibroblasts on a degradable scaffold that has been used clinically to increase the healing rates of skin ulcers (Mansbridge et al. 1998). Therefore, following the investigation of the impact of cryopreservation on the storage of MSCs, it is suggested that MSCs within a scaffold could be stored using the same method of cryopreservation, for example the MSC-HA combination as studied in my thesis. These tissue-engineered constructs could be stored by cryopreservation prior to implantation into patients commercially. However, this also requires further research.

6.1.4 Limitations of the methods used in this thesis

Although the cells isolated from bone marrow demonstrated properties of MSCs, it is unlikely that a truly homogenous population of cells were isolated.

Cell culture experimentation has specific limitations associated with it, which include the selection of cells that survive in these conditions. In the case of chondrocytes, this can lead to de-differentiation of cells, although in my experiments the cells continued to function demonstrated by protein production. Furthermore, when cells are grown on flat surfaces in cell culture, the cells become orientated in one direction, resulting in loss of 3-d structure. However, the use of an HA scaffold in Chapters 3 & 4 allowed the cells to grow in a 3-d environment resulting in the formation of extracellular matrix.

All the studies in my thesis were conducted *in vitro* therefore these results may not directly reflect cell behaviour that would occur *in vivo*. However, in the controlled environment of cell culture, the experiments of my thesis form the preliminary work for the use of MSCs for tissue engineering of bone. This forms the basis from which animal studies can be conducted before testing in clinical trials.

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